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13. ABSTRACT *(Maximum 200 Words)*

Recently we showed that flavonoid antioxidant silymarin inhibits erbB1 activation followed by a G1 arrest and inhibition of PCA cell growth. In this grant we proposed studies to define cause and effect relationship for this effect at membrane, cytoplasmic and nuclear levels, and define in vivo effect of silymarin on PCA tumor growth in nude mice. Treatment of LNCaP and DU145 human prostate carcinoma cells with silibinin (the pure form of silymarin) resulted in strong inhibition of ligand binding to erbB1 receptor and ligand internalization, and inhibition of erbB1 activation followed by dimerization. These inhibitory effects of silibinin also corroborate with its inhibitory effect on both cellular and released expression of TGFα in these two cell lines. In cytoplasmic signaling, silibinin showed strong decrease in ERK1/2 phosphorylation in both LNCaP and DU145 cells and inhibition in their growth. In nuclear signaling, silibinin showed strong increase in hyperphosphorylation of RB (in LNCaP) and related proteins (in DU145) with changes in E2F family of transcription factors. These alterations were identified to be due to modulation of cell cycle regulators such as CDKIs, CDKs and cyclin. Silibinin showed strong G1 arrest, and caused differentiation of both LNCaP and DU145 cells. In one pilot studies completed silymarin feeding to nude mice showed ~80% inhibition of DU145 tumor weight. Other studies also standardized both LNCaP and DU145 xenograft growth in nude mice, and PCNA and TUNEL staining in tumor samples. Our results suggest that silibinin could afford prevention/ intervention of human prostate cancer.

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5. INTRODUCTION

Subject: Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy and the second leading cause (after lung) of cancer deaths in American males (1,2). Several studies have suggested that diet and androgen play a major role in the pathogenesis as well as in the promotion of PCA (2-8). Since PCA growth and development is initially androgen-dependent, androgen deprivation is used to control PCA (9). However, within a few years, tumor re-growth occurs which is largely due to progression/selection of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (10). At this stage, PCA growth is causally dependent on enhanced expression of growth factors and their receptors which lead to an autocrine loop for uncontrolled PCA growth and metastatic potential (11-20). In this regard, an enhanced expression of epidermal growth factor receptor (EGFR) and other members of this family and related ligands have been shown with high frequency in PCA (11-20). **Thus, new approaches are needed to control advanced androgen-dependent and -independent PCA as well as to prevent the disease from developing. One approach to reduce PCA incidence and associated mortality is chemoprevention/chemo-intervention targeted towards the impairment of growth factor receptor-mediated membrane, cytoplasmic and nuclear signaling, and associated growth of malignant PCA cells**. Several studies suggest that micro-chemicals present in fresh fruit, yellow-green vegetables and various herbs reduce the human cancer incidence and mortality due to stomach, colon, breast, lung, bladder, esophageal, prostate and other cancers (21-31). Among these, polyphenolic antioxidants are receiving increased attention in recent years as cancer preventive/interventive agents (32-34). **Silymarin** is also a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum (L.) Gaertn*) and in its pure form composed mainly of stereoisomer **silibinin** (~90%, w/w) (35,36). For more than twenty-five years, silymarin and silibinin have been used clinically in Europe as an anti-hepatotoxic agent (37-40). In recent years, silymarin has also been used as a therapeutic agent in liver diseases in Asia and the United States, and marketed (in USA and Europe) as dietary supplement. Toxicity data on silymarin and silibinin as therapeutic agents show they are exceptionally well tolerated and largely free of adverse effects (41-44). **Together, we concluded that silymarin and silibinin are non-toxic and have been well studied as dietary supplements and as therapeutic plant flavonoids.**

Purpose: **The purpose of the studies in the current grant was the prevention/intervention of prostate cancer by silymarin targeted towards the impairment of EGFR-mediated mitogenic signaling involving the mechanistic approach on membrane receptor, cytoplasmic and nuclear signals and their biological significance in terms of PCA cell growth inhibition in nude mice tumor xenograft model.**

Scope: **An ideal cancer preventive agent should have a) little or no toxic effects; b) high efficacy; c) a known mechanism of action; d) low cost; and e) human acceptability (26). Therefore, we emphasize that silymarin (or silibinin) have promise and potential to be ideal cancer preventive/interventive agents against prostate cancer and based on our completed studies, as therapeutic agents for early recurrent disease.** The major scope of the studies in current grant is that their outcome will build a base for long-term phase II studies to a) further define the role of erbB family of RTKs and down stream events (e.g. MAPK/ERK1/2-mediated mitogenic signaling and alteration in cell cycle regulators) in human prostate cancer as molecular target(s) for intervention, and b) evaluate the therapeutic (and/or preventive) effects of silymarin (or silibinin) and other related agents against prostate cancer in investigative clinical trials with correlative laboratory studies.

6. BODY

Under this section "BODY", the research accomplishments associated with each **approved** Statement of Work are described in sufficient detail in terms of experimental design, method employed, data obtained, interpretation of the results, and conclusion(s) drawn from the research findings. All the data are presented and discussed irrespective of their positive or negative outcome in a given experiment performed. We would also like to highlight here that each **approved** Statement of Work, during entire funding period, is described in detail in following pages in the same order as proposed in the original plan.

Rationale for the studies proposed in the current grant: In addition to its exceptionally high anti-carcinogenic activity in different epithelial tumorigenesis protocols (45-47), in mechanistic studies we found that silymarin inhibits EGFR (erbB1) activation and induces anti-proliferative effects in epidermoid carcinoma cells A431 (48,49). As mentioned earlier, since erbB1 and other members of the erbB family play important roles in human prostate cancer (11-20), we reasoned that silymarin, by inhibiting erbB1 activation, may impair associated downstream events leading to growth inhibition of prostate cancer cells. In our preliminary studies (at the time of the submission of present grant) we showed that silymarin treatment of androgen-independent human prostate carcinoma DU145 cells results in a significant inhibition of transforming growth factor α (TGFα)-mediated activation of erbB1, but no change in its protein levels. Silymarin treatment also resulted in a significant decrease in tyrosine phosphorylation of an immediate down-stream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. Blocking the activation of erbB1 by silymarin was associated with a significant induction of cyclin-dependent kinase inhibitors (CDKIs) Cip1/p21 and Kip1/p27, concomitant with a decrease in cyclin-dependent kinase (CDK) 4 expression. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs together with a marked decrease in the kinase activity of CDKs and associated cyclins. Silymarin treatment also induced a G1 arrest, and resulted in a highly significant to complete inhibition of both anchorage-dependent and -independent growth of DU145 cells. The data from all these studies are published (prior to the current grant funding) in a journal article entitled "A flavonoid antioxidant silymarin inhibits activation of erbB1 signaling, and induces cyclin-dependent kinase inhibitors, G1 arrest and anti-carcinogenic effects in human prostate carcinoma DU145 cells" by Zi, X., Grasso, A.W., Kung, H.-J. and Agarwal, R.: Cancer Res., 58: 1920-1929, 1998.

Together, above summarized results suggested that silymarin may exert a strong anti-carcinogenic effect against prostate cancer, and that this effect is likely to involve impairment of erbB1-mediated signaling pathway, induction of CDKIs, and a resultant G1 arrest. This suggestion was the rationale for the proposed studies (in four aims/tasks) in the current funded grant.

6.1 Task (Aim) I: To study the effect of silymarin on membrane signaling, Months 1-9: The outcomes of these studies are described in detail below.

6.1.a Grow and maintain LNCaP and DU145 cells in culture: Both androgen-dependent LNCaP and androgen-independent DU145 human prostate carcinoma cell lines were obtained from American Type Culture Collection (Bethesda, MD). Both the cell lines were thawed quickly in a waterbath at 37oC, and seeded in T100 cell culture flask in PRMI 1640 culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (all cell culture materials were from Gibco BRL, Gaithersburg, MD). Cultures from both the cell lines were maintained under standard culture conditions at 37oC, 95% air and 5% CO_2, and 90-95% humidity, in the above described culture medium. The cultures grown and maintained under these conditions were employed in all the studies detailed later in this section.

Negative finding(s): Not applicable.

Methodological problems: We have had no methodological or any other problems in growing and maintaining the two cell lines.

6.1.b & c Assess the effect of silymarin on ligand binding to erbB1 and the internalization of the ligand in both cell lines: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' (obtained from Sigma Chemical Co, Milwaukee, WI) was used in the studies.

Experiment 1: Experimental design and Method: **First**, we did a dose-dependent study to assess the effect of silibinin on ligand binding to erbB1 and ligand internalization in both cell lines. These studies were performed as described by Baulida et al (50) with desired modifications. LNCaP and DU145 cells were seeded at 0.12 million cells/well in 12-well dishes under standard culture conditions, and after 24 hrs, the cells were subjected to serum starvation. Briefly, the attached cells were quickly washed two times with phosphate buffered saline (PBS) and replaced with fresh medium without serum. This serum starvation was necessary to shutdown the constitutive activation of erbB1 and to make the receptor available for the ligand binding. After 34 hrs under these serum starvation conditions, the cultures were treated with dimethyl sulfoxide (DMSO) vehicle alone or varying concentrations (50, 75, 100 and 150 μg/ml) of silibinin in DMSO. The final concentration of DMSO in each treatment including control was 0.5% (v/v) of the medium. Two hrs after these treatments, cultures were incubated with ^{125}I-epidermal growth factor (EGF) [2 ng (0.28 μCi)/ml, specific activity 900 Ci/m mol obtained from Amersham Pharmacia Biotech] at 37°C for 6 min. At the end, medium was aspirated, and cultures were rapidly washed with ice-cold medium. The surface bound ^{125}I-EGF was removed by a rapid wash with 0.2 ml of glacial acetic acid (pH 2.8), added to 5 ml scintillation fluid and quantitated as a measure of surface bound ligand. The cells were then solubilized in 0.2 ml of 1 M NaOH, added to 5 ml scintillation fluid and counted to determine internalized ^{125}I-EGF (ligand). The nonspecificity of binding and internalization of ligand was determined by adding 400 ng unlabeled EGF 5 min prior to hot ligand.

Results: As shown by data in **Figure 1**, silibinin treatment of LNCaP (panel A) and DU145 (panel B) cells resulted in a highly significant inhibition (in a dose-dependent manner) of both ligand binding to erbB1 as well as internalization of the ligand. When the results were analyzed for LNCaP cells (**Figure 1A**), silibinin treatment at 50 μg/ml dose showed very little inhibitory effect towards ligand binding to erbB1, however higher doses of 75, 100 and 150 μg/ml silibinin resulted in a 30, 50 and 75% inhibition ($P < 0.001$, Student's t test), respectively. In terms of ligand internalization in LNCaP cells, these four doses of silibinin resulted in much stronger effect accounting for 55, 70, 83 and 95% inhibition ($P < 0.001$, Student's t test) at 50, 75, 100 and 150 μg/ml doses, respectively. In case of DU145 cells (**Figure 1B**), a reverse trend was observed towards the inhibitory effect of silibinin on ligand binding to erbB1 and its internalization. In this case, silibinin treatment at 50, 75, 100 and 150 μg/ml doses resulted in 20, 35, 50 and 64% inhibition ($P < 0.05$ to 0.001, Student's t test) in ligand binding to erbB1, but 2, 12, 22 and 27% inhibition ($P < 0.1$ to 0.001, Student's t test) in ligand internalization, respectively. An analysis of the results for nonspecific binding study using 200 fold excess of cold ligand showed that it was with 5% of specific binding for hot ligand (data not shown).

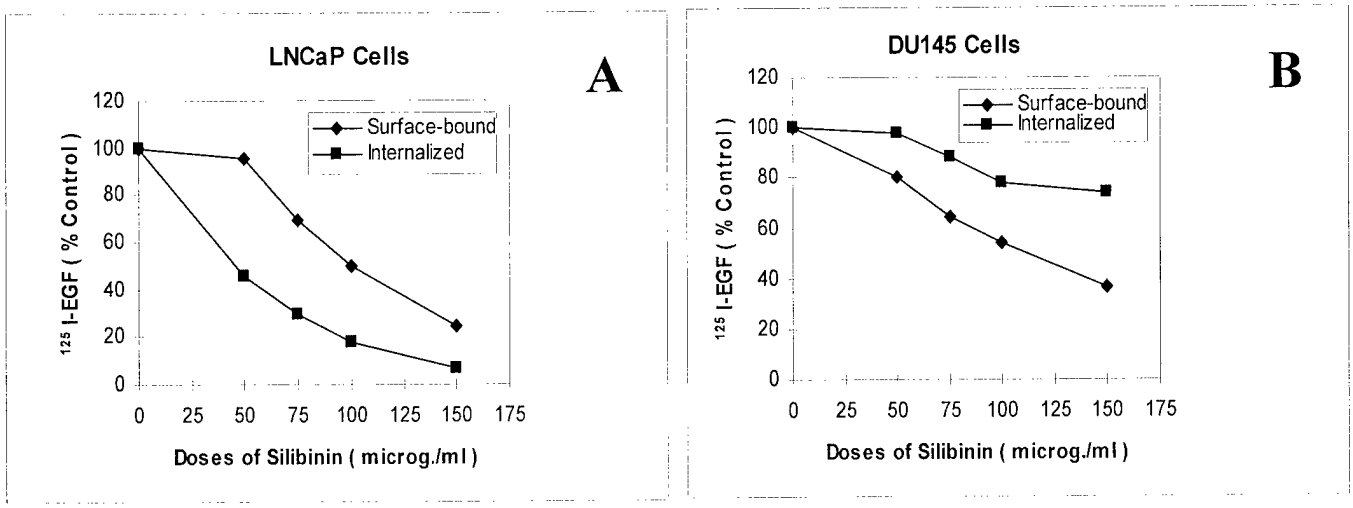

Figure 1: Dose-dependent inhibitory effect of silibinin on ligand binding to erbB1 and the internalization of the ligand in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are mean (with less than 5% error) of two independent experiment, each done in duplicate wells.

Experiment 2: Experimental design and Method: Based on the results from dose-dependent study detailed above, **second**, we did a time-response study to assess the effect of time of silibinin treatment on ligand binding to erbB1 and ligand internalization in both cell lines. The highest effective dose from **experiment 1**, 150 μg/ml silibinin was used in this set of experiment. LNCaP and DU145 cells were seeded at 0.12 million cells/well in 12-well dishes under standard culture conditions, and after 24 hrs, the cells were subjected to serum starvation as detailed above. After 34 hrs under these serum starvation conditions, the cultures were treated with DMSO vehicle alone or 150 μg/ml of silibinin in DMSO for 10 min (0.16 hr), 0.5, 8 or 16 hrs. After these treatments, cultures were incubated with ^{125}I-EGF (2 ng/ml) at 37°C for 6 min. At the end, medium was aspirated, and cultures were rapidly washed with ice-cold medium. The levels of surface bound ^{125}I-EGF and internalized ^{125}I-EGF (ligand) were then determined as described above.

Results: As shown by data in **Figure 2**, silibinin treatment, at 150 μg/ml dose, of LNCaP (panel A) and DU145 (panel B) cells resulted in a highly significant inhibition (in a time-dependent manner) of both ligand binding to erbB1 as well as internalization of the ligand. When the results were analyzed for LNCaP cells (**Figure 2A**), silibinin treatment for 10 min showed little inhibitory effect (15%) towards ligand binding to erbB1, however higher treatment times resulted in a 39, 64 and 74% inhibition ($P < 0.001$, Student's t test), respectively. In terms of ligand internalization in LNCaP cells, all the time points examined showed stronger effect accounting for 32, 44, 77 and 88% inhibition ($P < 0.001$, Student's t test) after 10 min, 0.5, 8 and 16 hrs of 150 μg/ml dose of silibinin treatment, respectively. In case of DU145 cells (**Figure 2B**), similar time-dependent inhibitory effect of silibinin on ligand binding to erbB1 and its internalization was observed. In this case, silibinin treatment at 150 μg/ml doses for 10 min, 0.5, 8 and 16 hrs resulted in 11, 50, 69 and 78% inhibition ($P < 0.05$ to 0.001, Student's t test) in ligand binding to erbB1, and 17, 34, 65 and 87% inhibition ($P < 0.05$ to 0.001, Student's t test) in ligand internalization, respectively.

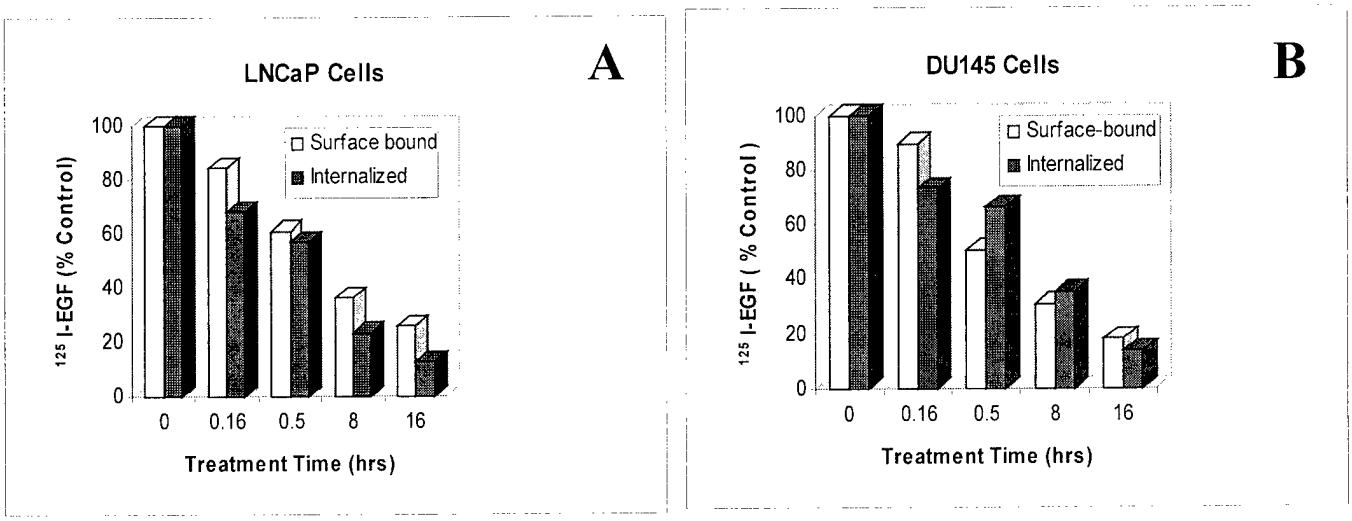

Figure 2: Time-dependent inhibitory effect of 150 μg/ml dose of silibinin on ligand binding to erbB1 and the internalization of the ligand in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are mean (with less than 5% error) of two independent experiment, each done in duplicate wells.

Taken together, the results shown in **Figures 1 and 2**, convincingly suggest that silibinin inhibits the binding of the ligand EGF to erbB1 receptor in both LNCaP and DU145 human prostate carcinoma cells that possibly also results in an inhibition of internalization of the ligand in these two prostate carcinoma cells. These findings also establish a cause for the observed inhibitory effect of silymarin (silibinin) on ligand (TGFa)-caused activation of erbB1 in DU145 cells reported recently by our group (51). It could be suggested that this effect of silymarin (silibinin) is directly due to its inhibitory effect on ligand binding to erbB1 and ligand internalization that leads to an inhibition of erbB1 activation.

Negative finding(s): In a dose-response study, the treatment of 34 hrs serum starved LNCaP and DU145 cells with 0.1, 1, 5, 10 and 25 μg/ml doses for 2 hrs followed by hot ligand treatment for 6 min, did not result in an inhibition of either ligand binding to erbB1 or ligand internalization (all these data are not shown). This experiment was done following same experimental strategy and methods as described above for **Experiment 1**.

Methodological problems: We have had no methodological or any other problems in performing the studies in these Tasks.

6.1.d *In vivo* and *in vitro* erbB1 intrinsic kinase activity assays evaluating the effect of silymarin in both cell lines: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

Experimental design and Method: We did a dose-dependent study to assess the *in vivo* effect of silibinin on erbB1 intrinsic kinase activity in both cell lines. These studies were performed as described recently by us (48) with desired modifications. LNCaP and DU145 cells were grown to 80% confluency in 100 mm dishes under the standard culture conditions detailed above, and treated with varying doses (10, 25, 50, 75, 100 and 150 μg/ml) of silibinin in DMSO or DMSO vehicle alone. Sixteen hrs after these treatments, medium was removed, cultures were washed with ice cold PBS, and cell lysates were prepared under native lysis conditions. Briefly, 0.5 ml lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40 and 0.2 U/ml aprotonin) was added per plate. After 15 min in lysis buffer at 4°C, the cell lysate was scraped from the plate, collected in microcentrifuge tubes and left on ice for additional 15 min followed by centrifugation, and clear supernatant was collected as soluble cell lysate for the desired studies. Equal amount of protein (200 μg/sample lysate) was subjected to immunoprecipitation with anti-EGFR antibody (clone 528 from Neomarkers, Union City, CA) and protein A beads as detailed by us recently (48,51). The immunoprecipitated erbB1 embedded in protein A beads was suspended in 25 μl of kinase assay buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 10 mM $MnCl_2$ and 1 mM $MgCl_2$) containing 10 μCi of [^{32}P]-ATP (from Amersham). The reaction mixture was incubated at 4°C for 15 min, terminated by adding 5 μl of 6 x sample buffer followed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% gel. The gel was dried followed by autoradiography.

Results: The cytoplasmic domain of erbB1 contains tyrosine kinase domain that is phosphorylated causing an activation of the receptor for further events (52). Recent studies from our laboratory have shown that treatment of A431 cells with silymarin results in a dose-dependent inhibition of intrinsic kinase activity of tyrosine in cytoplasmic domain of erbB1 suggesting this pathway as one of mechanisms of silymarin's effect on the inhibition of erbB1 activation (48). Interestingly, treatment of LNCaP and DU145 cells with different doses of silibinin for 16 hrs did not result in any inhibition of erbB1 intrinsic kinase activity in both the cell lines examined (**Figure 3**). In case of LNCaP cells (**Figure 3A**), compared to vehicle treated control, the lowest dose assessed (10 μg/ml silibinin) showed no change in receptor kinase activity. The higher doses examined (25-150 μg/ml), however, showed an increase in intrinsic kinase activity (**Figure 3A**). Unlike the results with LNCaP cells, in case of DU145 cells (**Figure 3B**), there was no considerable change in kinase activity following silibinin treatment at different doses. Together, these findings suggest that unlike A431 cells, in case of prostate carcinoma LNCaP and DU145 cells, the inhibitory effect of silibinin of erbB1 activation does not involve its effect on receptor tyrosine kinase activity inhibition.

Negative findings: As discussed above in the result section and shown in **Figure 3**, the data obtained were in contrast to our anticipation, and showed that silibinin does not inhibit erbB1 intrinsic kinase activity in either of LNCaP and DU145 cells examined, and in fact causes some increase in the activity at higher doses in LNCaP cells. A cause for such an increase in kinase activity by silibinin in LNCaP cells remains to be studied.

Methodological problems: We have had no methodological or any other problems in performing the studies in this Task. In fact the positive A431 cell lysate sample and negative sample without any cell lysate were useful in confirming the validity of the assay in these studies (**Figure 3**).

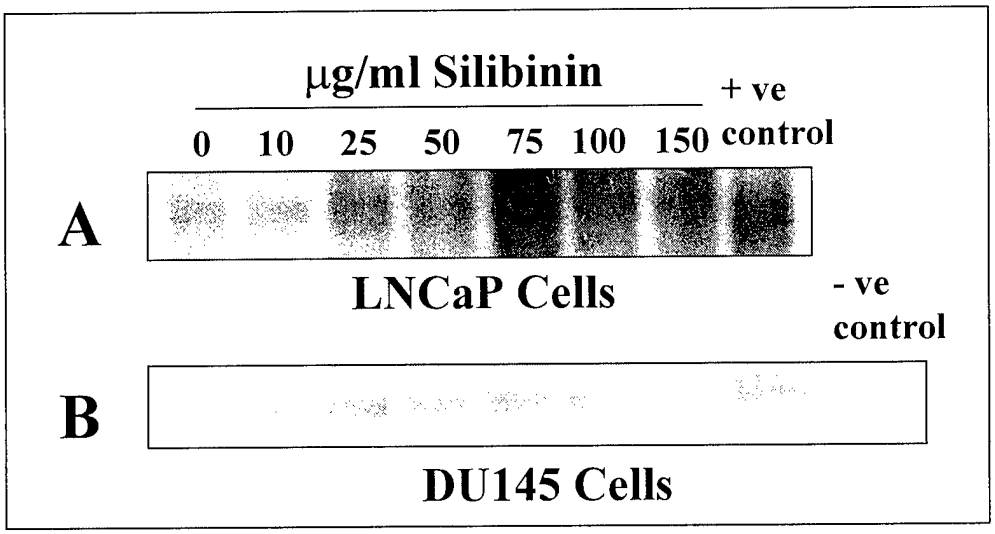

Figure 3: A lack of effect of silibinin on *in vivo* erbB1 intrinsic kinase activity in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are representative of three independent experiments with similar results. + ve control, cell lysate from A431 human epidermoid carcinoma cells that contain high erbB1 levels (48) was used in place of sample extract in the assay; - ve control, no cell lysate was used in the assay.

6.1.e erbB1 dimerization studies in intact cells and membrane preparation to assess the effect of silymarin: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

Experimental design and Method: We did a dose-dependent study to assess the inhibitory effect of silibinin on erbB1 dimerization in both cell lines. These studies were performed as described by Cochet et al (53) with desired modifications. LNCaP and DU145 cells were grown in 35 mm dishes to 80% confluency and then serum starved for 36 hrs. Cells were then treated with DMSO vehicle alone or varying concentrations of silibinin (50, 75, 100 and 150 μg/ml) for 2 hrs followed by ligand stimulation (50 ng/ml of TGFα,obtained from Gibco BRL) for 10 min at 37°C. The cross-linker, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDAC, obtained from Sigma Chem. Co.), was then added at 15 mM concentration for 15 min, and then cell lysates were prepared under non-denaturing conditions as described above. Equal protein (100 μg) from each cell lysate was subjected to SDS-PAGE on 4% gel followed by Western blotting. Membranes were probed with anti-EGFR and anti-phosphotyrosine antibodies followed by desired secondary antibodies and detection by ECL system as described recently by us (51).

Results: As shown by data in **Figure 4**, compared to vehicle treated controls, treatment of serum starved LNCaP and DU145 cells with different doses of silibinin followed by ligand stimulation resulted in a dose-dependent inhibition of ligand-caused activation of erbB1 in both the cell lines examined. As reported earlier that ligand-caused activation of erbB1 results in its dimerization leading to a new band at 340 kDa (53), we also observed the formation of this band in positive control samples from both the cell lines where serum starved cultures were treated with ligand for 10 min followed by EDAC for 15 min (**Figure 4, lane 3 in each panel**). Whether assessed in terms of erbB1 expression or tyrosine-phosphorylated erbB1, the formation of 340 kDa band following ligand stimulation was at the expense of a decrease in 170 kDa erbB1 which further confirms that 340 kDa band was dimerized erbB1 (**Figure 4, lane 3 versus lane 2 in each panel**). Consistent with its effect on inhibition of ligand-caused activation of erbB1, silibinin also showed a concentration-dependent inhibition of ligand-caused erbB1 dimerization at all the doses examined in both LNCaP and DU145 cells (**Figure 4**).

Negative finding(s): We did not get any negative results in this experiment. In fact, the data obtained were anticipated based on the findings from Tasks 'b' and 'c' showing that silibinin inhibits ligand binding to

erbB1 and ligand internalization. These effects of silibinin were anticipated to result in an inhibition of erbB1 activation followed by inhibition in erbB1 dimerization, which is a receptor activation-dependent phenomenon.

Methodological problems: We have had two major methodological problems in performing the studies in this Task. Firstly, treatment of both the cell lines during the last step with EDAC for erbB1 dimerization for 15 min turn out to be toxic for the cells. This problem is being handled by lowering the concentration of EDAC to 5 or 10 mM and reducing its treatment time to 5 or 10 min. In these scenarios, however, there was much reduced dimer formation. The second major problem encountered in this Task was separating a clear band at 340 kDa. As is evident from the data shown in **Figure 4**, it is almost impossible to get a clean crisp 340 kDa band from the samples where EDAC was used for receptor dimerization. A strong background (like protein trailing) was always evident in these samples. Further standardization of the method and technique is in progress to overcome these two problems.

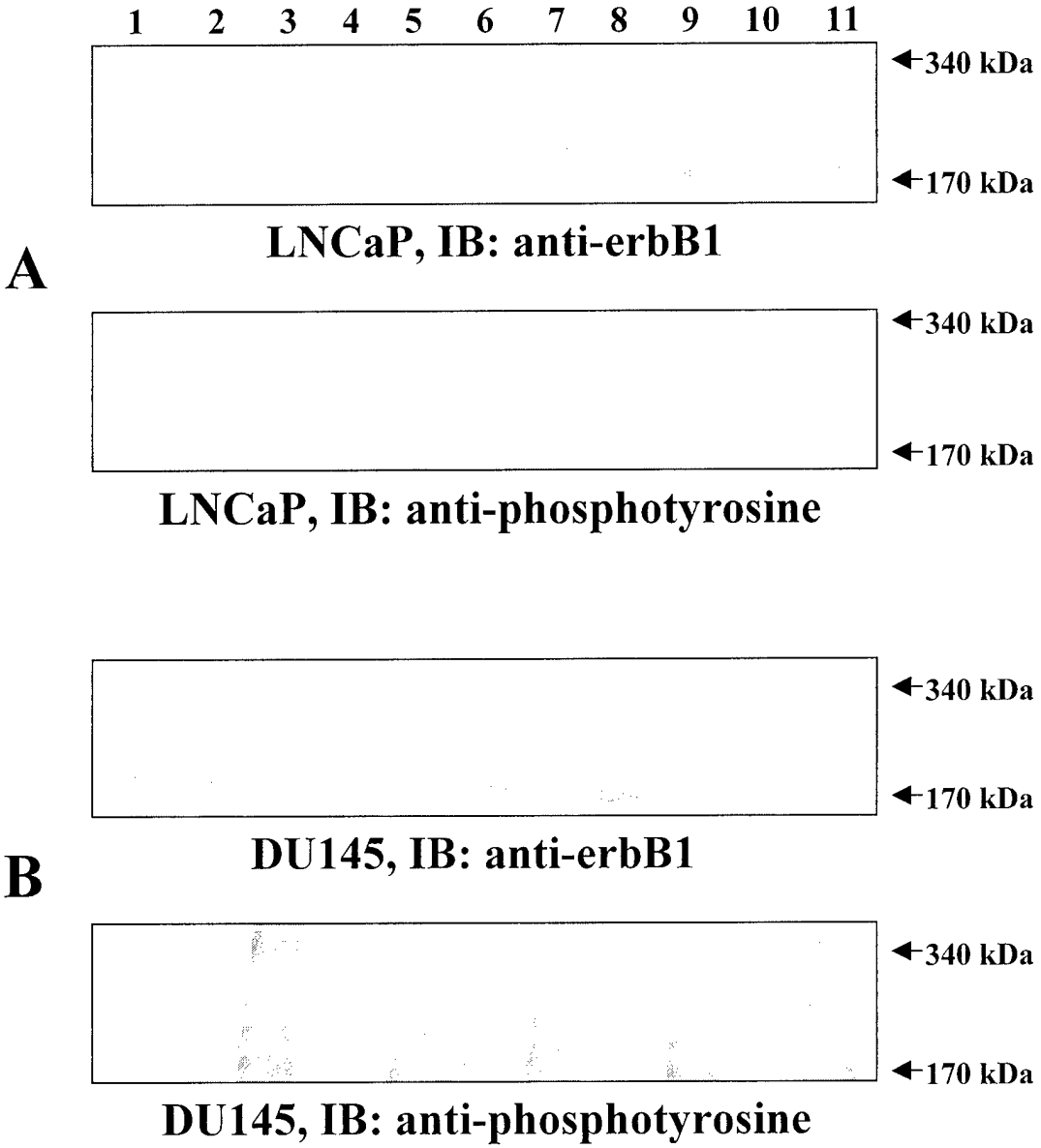

Figure 4: Inhibitory effect of silibinin on ligand-caused erbB1 dimerization in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In each case, the data shown are representative of three independent experiments with similar results. Lanes **1**, serum starved cells; **2**, serum starved cells treated with TGFα; **3**, same as lane 2 but with EDAC; **4, 6, 8 & 10**, same as lane 2 but treated with 50, 75, 100 & 150 μg/ml silibinin for two hrs prior to ligand stimulation; **5, 7, 9 & 11**, same as lanes 4, 6, 8 & 10 but with EDAC. IB, immunoblotting.

6.1.f & g Evaluate the effect of silymarin on TGFα release and TGFα expression in both LNCaP and DU145 cells: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

Experimental design and Method: We did both dose- and time-dependent study to assess the inhibitory effect of silibinin on TGFα release (the secreted form in the medium) and TGFα expression (cellular levels in the cells) in both cell lines. LNCaP and DU145 cells were grown to 60% confluency in 60 mm dishes under standard culture conditions detailed above. At this point, cultures were treated with DMSO vehicle alone or varying concentrations of silibinin (25, 50, 75 and 100 μg/ml) for 0, 6, 12, 24, 48 and 72 hrs. After these treatments, medium was collected from each dish and stored at –80oC till further assay. At the same time, cells were also collected, washed in ice cold PBS, and cellular extracts were prepared following step-by-step protocol provided by the manufacturer for TGFα ELISA assay kit (cat #QIA 61 from Oncogene Research Products, Cambridge, MA). Employing medium and cellular samples collected and prepared above, released (secreted) and cellular TGFα levels were determined using the ELISA kit and following the protocol provided with the kit.

Results: Several experiments are done in this Task, and a number of important findings were obtained from them. As shown by data in **Figure 5A**, in case of LNCaP cells, the cell growth alone in the absence of any silibinin treatment showed time-dependent increase in TGFα release in medium. Compared to a zero time point data, 6 hrs cell culture resulted in a 12-fold increase in ligand release which doubled by 24 hrs of culture; maximum TGFα release was observed following 48 hrs of LNCaP culture (**Figure 5A**). This observation was consistent no matter the data were analyzed in terms of amount of ligand/ml medium or per 10^6 cell. In the studies assessing the effect of silibinin on TGFα release in LNCaP cells, as shown by data in **Figure 5A**, it showed a highly significant inhibition in both dose- and time-dependent manner. Maximum effect of silibinin was evident following 48 hrs of its treatment and at the dose of 100 μg/ml (when the data are analyzed in terms of amount of ligand/10^6 cells) (**Figure 5A**). In terms of its effect towards ligand release/ml medium, silibinin showed much stronger inhibition even at lower doses and at other time points (**Figure 5A**).

Similar results were also observed in case of DU145 cells where culture growth alone over the period of time showed strong increase in TGFα release in the medium with optimum levels at 48 hrs followed by a small decline at 72 hrs (**Figure 5B**). When the effect of silibinin was assessed on ligand release in DU145 cells, compared to that observed in LNCaP cells, much stronger inhibitory effect was evident that was also dependent on silibinin dose and time of its treatment (**Figure 5B**). Once again, the maximum inhibition was evident at 100 μg/ml silibinin dose but was at 24 hrs of treatment (when the data are analyzed in terms of amount of ligand/10^6 cells) (**Figure 5B**). In terms of ligand release/ml of medium, even lower doses of silibinin showed strong inhibition (**Figure 5B**). Taken together, the findings from both LNCaP and DU145 cells convincingly suggest that silibinin inhibits the release of TGFα, and that this inhibition is not due to a decrease in total number of cells in silibinin treated samples.

Conversely to ligand release studies discussed above and shown in **Figure 5**, in case of cellular expression of TGFα, 6 to 72 hrs following initial culture, no significant change in its level was evident in either of the two prostate carcinoma cells examined in terms of both TGFα levels/μg cellular protein or /10^6 cells (**Figure 6**). A very strong inhibitory effect of silibinin, however, was observed on the cellular levels of TGFα in both LNCaP and DU145 cells (**Figure 6**). In case of LNCaP cells (**Figure 6A**), the inhibitory effect of silibinin was as high as 80% at both 75 and 100 μg/ml doses following 72 hrs of treatment; almost comparable inhibition was also evident at these doses after 48 hrs of treatment (**Figure 6A**). Even lower doses of silibinin showed strong inhibition following 24 hrs of treatment (**Figure 6A**). Similarly, silibinin also showed strong inhibitory effect on cellular TGFα expression in DU145 cells (**Figure 6B**). In this case, as high as 73% inhibition was observed at 50, 75 and 100 μg/ml silibinin doses after 72 hrs of treatment and 55-65% inhibition after 48 hrs of treatment at same doses (**Figure 6B**). Significant inhibitory effect of silibinin was also observed 24 hrs following its treatment at these doses and accounted for 37-41% inhibition (**Figure 6B**). Taken together, these findings from both LNCaP and DU145 cells corroborate those obtained for secreted TGFα, and suggest that silibinin inhibits the cellular levels of TGFα that results in a decrease in TGFα release.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

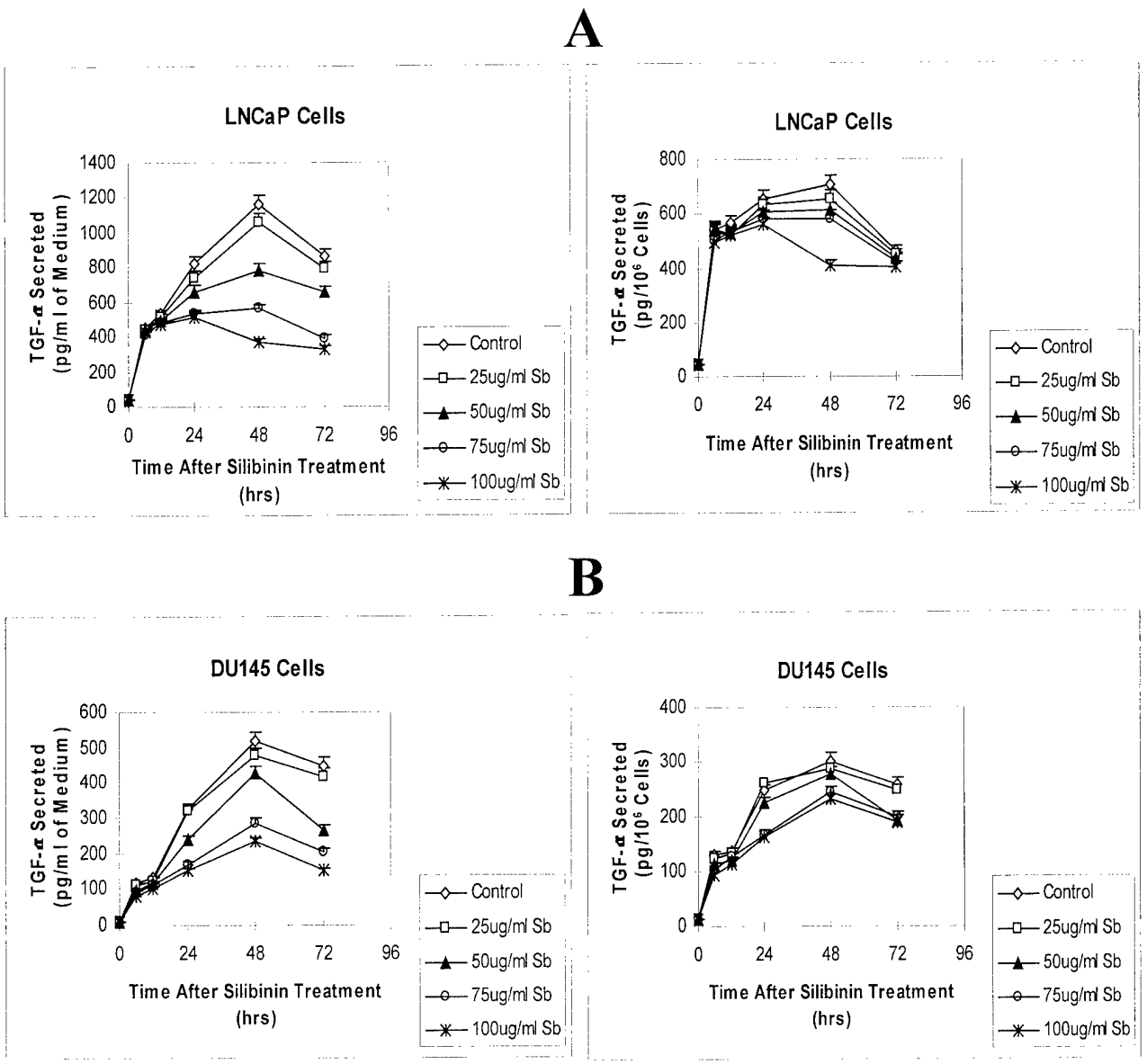

Figure 5: Dose- and time-dependent inhibitory effect of silibinin on TGFα release (in medium) in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In order to show the significance of the observed inhibitory effect of silibinin on TGFα release in both the cell lines, the data are calculated as the amount of TGFα (in pg) secreted in 1 ml of medium, and the amount of TGFα secreted by 10^6 cells following vehicle (control) treatment or different doses of silibinin treatment. The later calculation was important because it can be argued that since silibinin treatment inhibits the growth of cells, a decrease in the observed TGFα release is due to a decrease in total number of cells in silibinin treated cases. However, as can be seen from the data presented, after the correction for cell numbers, that is not the case. Each data point shown is the mean (± SE) of two independent experiment each done in triplicate.

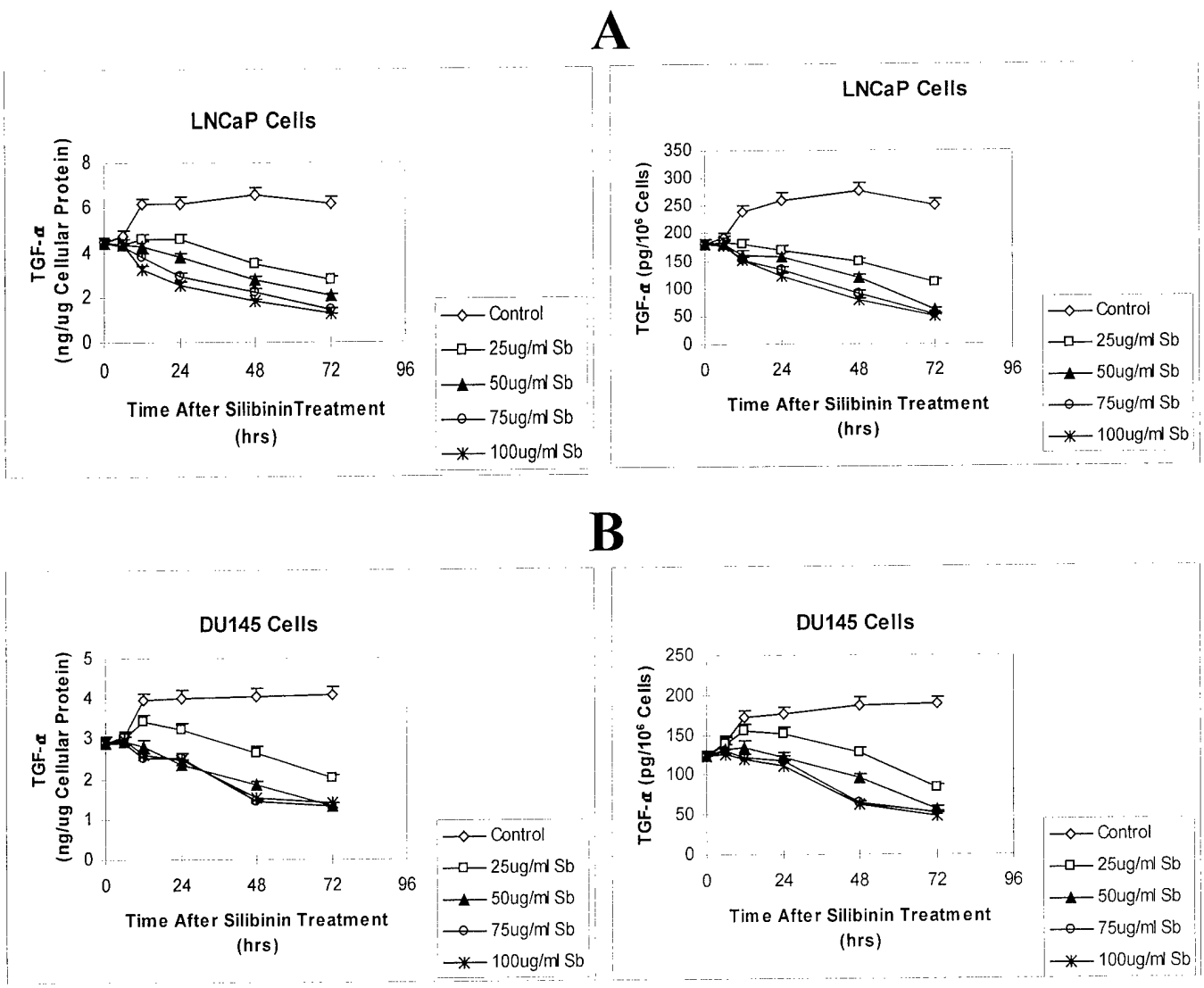

Figure 6: Dose- and time-dependent inhibitory effect of silibinin on cellular TGFα expression in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above. In order to show the significance of the observed inhibitory effect of silibinin on cellular TGFα expression in both the cell lines, the data are calculated as the amount of TGFα (in ng) per 1 μg of cellular protein, and the amount of TGFα per 10^6 cells following vehicle (control) treatment or different doses of silibinin treatment. Both the calculations lead to comparable pattern, and suggest that the observed inhibitory effect of silibinin on TGFα release be due to a highly significant inhibition of cellular TGFα expression following silibinin treatment. Each data point shown is the mean (± SE) of two independent experiment each done in triplicate.

6.2 **Task (Aim) II: To study the effect of silymarin on cytoplasmic signaling, Months 9-18:** The outcomes of these studies are described in detail below.

6.2.a & c Assess the effect of silymarin on activation of erbB1 in LNCaP and DU145 cells: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies.

Experiment 1. Experimental design and Method: Based on the findings shown in **Figure 1** that 150 μg/ml dose of silibinin results in a highly significant inhibition of ligand binding to erB1 as well as ligand internalization, in the present experiment, we used same dose of silibinin to assess its time-dependent inhibitory effect on erbB1 activation in both LNCaP and DU145 cells. LNCaP and DU145 cells were grown to 60% confluency in 100 mm dishes under standard culture conditions detailed above. At this point, cultures were treated with DMSO vehicle alone or 150 μg/ml dose of silibinin in DMSO. After 6, 12, 24, 48 and 72 hrs., medium was removed, cultures were washed two times with ice cold PBS, and cell lysates were prepared under non-denaturing conditions as described above in detail. Equal amount of protein (200 μg) from each cell lysate was diluted to 1 ml with lysis buffer and added with 2 μg of anti-EGFR (erbB1) antibody (from UPSTATE Biotechnology, Lake Placid, NY) followed by rotating this mixture at 4°C for 4 hrs. Thereafter, 25 μl of protein A agarose beads were added, and this mixture was incubated overnight at 4°C. The next day, beads were collected by centrifugation, washed four times with lysis buffer and the immunoprecipitated erbB1 was denatured with 30 μl of 1x SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on 8% gel, and separated proteins were transferred on to nitrocellulose membrane by Western blotting. The membranes were probed with anti-phosphotyrosine and anti-EGFR antibodies (from UPSTATE Biotechnology, Lake Placid, NY) followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system.

Results: As expected, treatment of both LNCaP and DU145 cells with silibinin resulted in a highly significant inhibition (in a time-dependent manner) of constitutive erbB1 activation (**Figure 7**). In case of LNCaP cells, as shown in **Figure 7A**, compared to DMSO treated control, silibinin treatment at 150 μg/ml dose resulted in the inhibition of erbB1 activation as early as by 6 hrs. By 48 hrs most of activated erbB1 expression was inhibited, and by 72 hrs it was not detectable. The observed inhibitory effect of silibinin of erbB1 activation (tyrosine phosphorylation) was not due to a decrease in total erbB1 protein expression as evident by a no change in its content following silibinin treatment for the time points studied (**Figure 7A, lower panel**). In case of DU145 cells, much stronger inhibitory effect of silibinin at this dose was evident on erbB1 activation (**Figure 7B**). Whereas 6 hrs of silibinin treatment was not effective, by 12 and 24 hrs, most of activated erbB1 expression was inhibited, and by 48 hrs it was not detectable (**Figure 7B**). Once again, this effect of silibinin in DU145 cells was not due to a change in total erbB1 protein levels (**Figure 7B, lower panel**). Together, these results convincingly suggest that silibinin inhibits erbB1 activation in both LNCaP and DU145 human prostate carcinoma cells, and that this effect is due to: **a)** inhibition of ligand binding to erbB1 followed by an inhibition in ligand internalization, and **b)** inhibition in cellular expression of TGFα followed by a decrease in its release.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

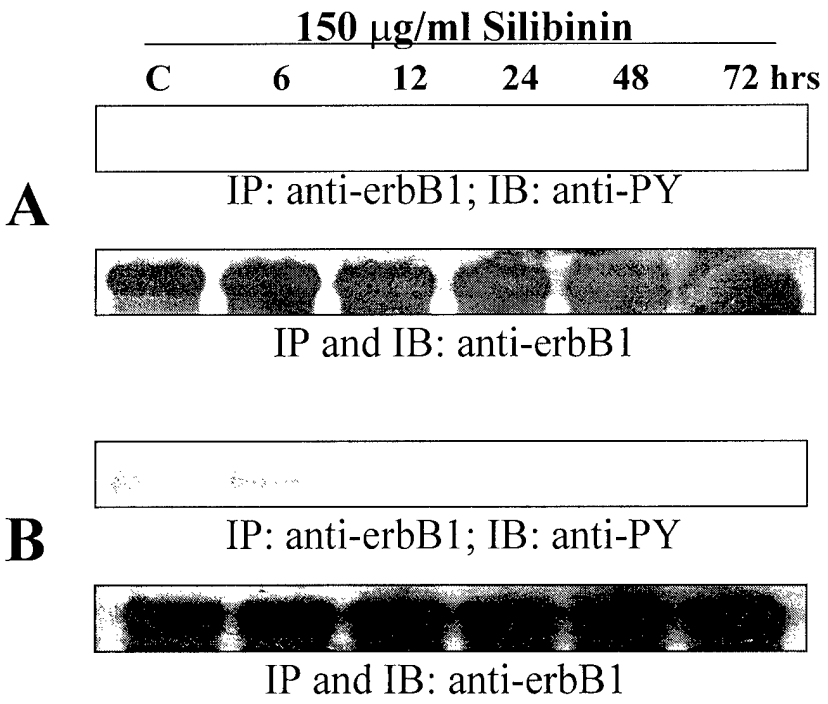

Figure 7: Time-dependent inhibitory effect of silibinin on constitutive erbB1 activation in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above, and the treatments are labeled as such in the figure shown; C, vehicle treated control at 36 hrs. In each case, the data shown are representative of three independent experiments with similar results. IP, immunoprecipitation; IB, immunoblotting.

Experiment 2. Experimental Design and Method: We next also performed the studies to assess the effect of silibinin on ligand caused activation of erbB1 in both LNCaP and DU145 human PCA cells. For these studies, 60% confluent LNCaP and DU145 cultures were washed twice with PBS, and then starved in serum free medium for 36 hrs with one serum free medium change after 20 hrs. During the last 2 hrs of starvation, the cultures were treated with DMSO or various doses of silibinin (50, 75 and 100 μg/ml of medium) in DMSO. At the end of these treatments, cultures were added with either PBS alone or EGF (50 ng/ml of medium) and incubated for 15 min at 37°C. Thereafter, medium was aspirated, cultures were quickly washed two times with cold PBS and cell lysates were prepared. For erbB1 activation studies, 400 μg of protein lysate per sample was subjected to immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting. Membranes were probed with anti-phosphotyrosine or anti-EGFR antibody (Upstate Biotechnology, Lake Placid, NY) followed by peroxidase conjugated appropriate secondary antibody and visualization by enhanced chemiluminescence detection system.

As shown in Figure 8, treatment of serum starved LNCaP and DU145 cells with silibinin for 2 hrs followed by EGF resulted in a strong inhibition of erbB1 activation. In case of LNCaP cells (Fig. 8A, upper panel), compared to serum starved cultures showing complete diminution of erbB1 activation, treatment of starved cells at 50 ng/ml EGF resulted in a strong activation of erbB1. Pretreatment of serum starved cells with 50, 75 and 100 μg/ml doses of silibinin for 2 hrs followed by similar EGF treatment resulted in a strong inhibition of ligand-caused erbB1 activation. Densitometric analysis of the blots from three independent studies showed that the observed inhibition was statistically significant and accounted for 58, 73 and 75% decrease in phosphorylated erbB1 at 50, 75 and 100 μg/ml doses of silibinin, respectively, when compared to EGF alone treated sample (Fig. 8A, upper panel). In case of DU145 cells, silibinin pre-treatment also resulted in a statistically significant inhibition of EGF-caused erbB1 activation (Fig. 8B, upper panel). In this case, however, the effect was more dose-dependent where 50, 75 and 100 μg/ml doses showed 40%, 65% and complete inhibition of EGF-caused erbB1 activation, respectively (Fig. 8B, upper panel). In both LNCaP and DU145

cells, the observed inhibition in erbB1 activation was not due to a decrease in erbB1 protein levels (Figs. 8A and 8B, lower panels) suggesting a possibility of direct association of inhibition of ligand binding with the observed inhibition in erbB1 activation by silibinin.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

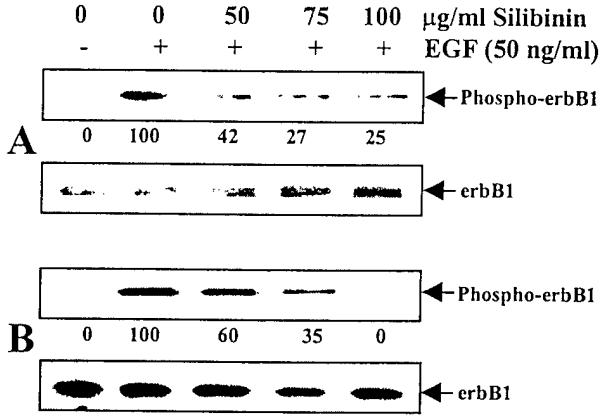

Figure 8. Effect of silibinin on ligand-caused erbB1 activation. LNCaP (panel A) and DU145 (panel B) cells at 60% confluency were serum starved for 36 hrs, and during last 2 hrs of starvation, treated with DMSO or various doses of silibinin in DMSO. Cultures were then treated with either PBS alone or EGF (50 ng/ml of medium) for 15 min at 37°C. Cell lysates were prepared, and erbB1 was immunoprecipitated followed by SDS-PAGE and Western blotting. Membranes were probed with anti-phosphotyrosine (upper panels in A and B) or anti-EGFR (lower panels in A and B) antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. The treatment in each lane is as marked in the figure.

6.2.b & d Assess the effect of silymarin on MAPK activation in LNCaP and DU145 cells: For all the studies performed assessing the effect of silymarin on different molecular events in LNCaP and DU145 cells, the pure form of silymarin, namely 'silibinin' was used in the studies. The MAPK activation studied was ERK1/2.

Experiment 1. Experimental design and Method: Based on the findings shown in **Figure 1** that 150 µg/ml dose of silibinin results in a highly significant inhibition of ligand binding to erB1 as well as ligand internalization, and **Figure 7** showing that this dose of silibinin significantly inhibits constitutive erbB1 activation in a time-dependent manner, in the present experiment, we used same dose of silibinin to assess its time-dependent inhibitory effect on MAP/ERK1/2 activation in both LNCaP and DU145 cells. LNCaP and DU145 cells were grown to 60% confluency in 100 mm dishes under standard culture conditions detailed above. At this point, cultures were treated with DMSO vehicle alone or 150 µg/ml dose of silibinin in DMSO. After 12, 24, 48 and 72 hrs., medium was removed, cultures were washed two times with ice cold PBS, and cell lysates were prepared under non-denaturing conditions as described above in detail. Equal amount of protein (80 µg) from each cell lysate was denatured with SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on 12% gel, and separated proteins were transferred on to nitrocellulose membrane by Western blotting. The membranes were probed with anti-phospho MAPK/ERK1/2 and anti-MAPK/ERK1/2 antibodies (from New England Biolab Inc, Beverly, MA) followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system.

Results: Similar to our anticipation, treatment of both LNCaP and DU145 cells with silibinin resulted in a highly significant inhibition (in a time-dependent manner) of constitutive MAPK/ERK1/2 activation (**Figure 9**). In case of LNCaP cells, as shown in **Figure 9A**, compared to DMSO treated control, silibinin treatment at 150 μg/ml dose resulted in a strong inhibition of MAPK activation after 48 hrs and by 72 hrs it was not detectable. The observed inhibitory effect of silibinin on MAPK/ERK1/2 activation (tyrosine phosphorylation) was not due to a decrease in total MAPK/ERK1/2 protein expression as evident by a no change in its content following silibinin treatment for the time points studied (**Figure 9A, lower panel**). In case of DU145 cells, much stronger inhibitory effect of silibinin at this dose was evident on MAPK activation (**Figure 9B**). Whereas 12 and 24 hrs of silibinin treatment showed small inhibitory effect, by 48 hrs, most of activated MAPK/ERK1/2 expression was inhibited, and by 72 hrs it was almost not detectable (**Figure 9B**). This effect of silibinin in DU145 cells was not due to a change in total MAPK/ERK1/2 protein levels (**Figure 9B, lower panel**). Together, these results convincingly suggest that silibinin inhibits erbB1 activation in both LNCaP and DU145 human prostate carcinoma cells, and that this effect leads to a significant inhibition of mitogenic signaling mediated downstream by MAPK/ERK1/2.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

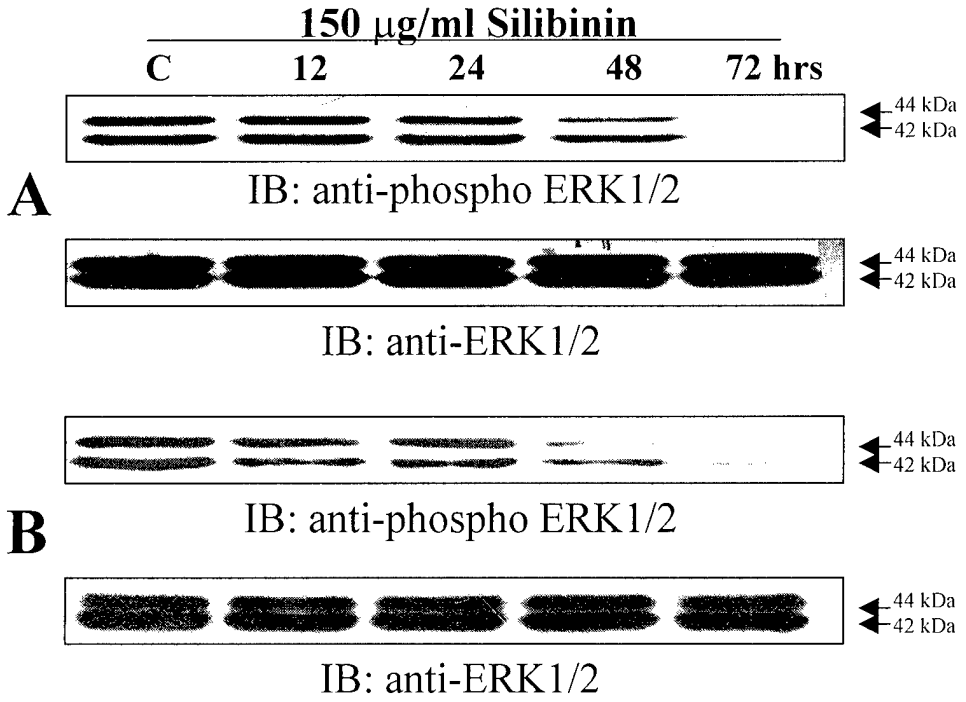

Figure 9: Time-dependent inhibitory effect of silibinin on constitutive MAPK/ERK1/2 activation in LNCaP (A) and DU145 (B) human prostate carcinoma cells. The details of experimental protocol and method are described above, and the treatments are labeled as such in the figure shown; C, vehicle treated control at 36 hrs. In each case, the data shown are representative of three independent experiments with similar results. IP, immunoprecipitation; IB, immunoblotting.

Experiment 2. Experimental Design and Method: We next also performed the studies to assess the effect of silibinin on ligand caused activation of MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells. For these studies, 60% confluent LNCaP and DU145 cultures were washed twice with PBS, and then starved in serum free medium for 36 hrs with one serum free medium change after 20 hrs. During the last 2 hrs of starvation, the cultures were treated with DMSO or various doses of silibinin (50, 75 and 100 μg/ml of medium)

in DMSO. At the end of these treatments, cultures were added with either PBS alone or EGF (50 ng/ml of medium) and incubated for 15 min at 37°C. Thereafter, medium was aspirated, cultures were quickly washed two times with cold PBS and cell lysates were prepared. For ERK1/2 activation studies, 50 µg of protein lysate per sample was denatured with 2x sample buffer, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gel, and separated proteins were transferred on to membrane by Western blotting. Membranes were probed with anti-phospho ERK1/2 and anti-ERK1/2 antibodies (from New England Biolab Inc, Beverely, MA) followed by desired secondary antibodies and detection by enhanced chemiluminescence system.

As shown in Figure 10, consistent with its inhibitory effect on erbB1 membrane signaling, treatment of LNCaP and DU145 cells with silibinin resulted in a significant inhibition of EGF-caused ERK1/2 activation. In case of LNCaP cells, the densitometric analysis of the blots from three independent studies (Fig. 10A, upper panel) revealed that 50, 75 and 100 µg/ml doses of silibinin pre-treated for 2 hrs followed by EGF stimulation result in 50, 38 and 45% decrease in phospho-ERK1 and 30, 40 and 30% decrease in phospho-ERK2 levels, respectively. In case of DU145 cells, however, similar silibinin treatments resulted in 50, 60 and 80% decrease in phospho-ERK1 and 20, 50 and 45% decrease in phospho-ERK2 levels, respectively, as compared to EGF alone treated samples (Fig. 10B, upper panel). Similar to inhibition of erbB1 activation, in both LNCaP and DU145 cells, the observed decrease in phospho-ERK1/2 levels was not due to a decrease in ERK1/2 protein levels (Figs. 10A and 4B, lower panels) suggesting a possibility of direct association of inhibition of ligand binding with the inhibition of erbB1 activation followed by a decrease in phospho-ERK1/2 by silibinin.

Negative finding(s): We did not get any negative results in these studies.

Methodological problems: We have had no problems in performing the studies in these Tasks.

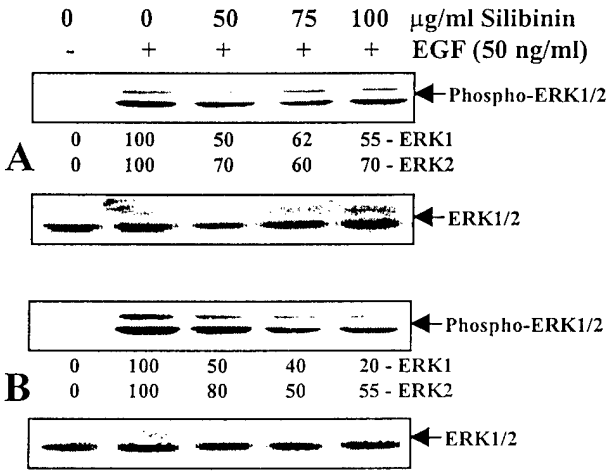

Figure 10. Effect of silibinin on ligand-caused ERK1/2 activation. LNCaP (panel A) and DU145 (panel B) cells at 60% confluency were serum starved for 36 hrs, and during last 2 hrs of starvation, treated with DMSO or various doses of silibinin in DMSO. Cultures were then treated with either PBS alone or EGF (50 ng/ml of medium) for 15 min at 37°C. Cell lysates were prepared, and 50 µg of protein lysate per sample was subjected to SDS-PAGE and Western blotting as detailed in Methods. Membranes were probed with anti-phospho-ERK1/2 (upper panels in A and B) or anti-ERK1/2 (lower panels in A and B) antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. The treatment in each lane is as marked in the figure.

6.3 Task (Aim) III: To study the effect of silymarin on nuclear signaling, Months 15-24: The outcomes of these studies are described in detail below.

6.3.a & b Assess the effect of silymarin on RB and related proteins and E2F levels and binding in LNCaP and DU145 cells: For all the studies, the pure form of silymarin, namely "silibinin" was used.

Experiment 1: Design and Methods: First we conducted studies in DU145 human PCA cells. For such studies, DU145 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions. 60% confluent cultures were treated with either dimethyl sulfoxide (DMSO) alone or different doses of silibinin (50, 100 or 200 μM) in DMSO for 6, 12 and 24 hrs. The final concentration of DMSO in culture medium during silibinin treatment did not exceed 0.1% (v/v), and therefore, same concentration of DMSO was present in control dishes. After these treatments, medium was aspirated, cells were washed two times with cold PBS, and cell lysates were prepared. For western immunoblotting, 40-60 μg of protein lysate per sample was denatured with 2x-sample buffer, samples were subjected to SDS-PAGE on 6, 12 or 16% gel, and separated proteins were transferred onto membrane by Western blotting. The levels of RB/p107, RB2/p130, E2F3 and E2F5 were determined using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody and ECL detection. We would like to highlight here that same sample lysates were also used to determine CDK2, CDK4, cyclin D1, cyclin E, Cip1/p21, Kip1/p27, p15, p16, p18 and p19 protein levels (as proposed in task IIIc) for a direct comparison and establishing a cause and effect relationship.

Western blot analysis showed that silibinin treatment of DU145 cells resulted in a strong increase in hypophosphorylated forms of RB/p107 and RB2/p130 (Fig. 11). In case of Rb/p107, as compared to DMSO treated control, 6 hrs silibinin treatment caused 1.7-2.3-fold increase in the levels of hypophosphorylated Rb/p107 (Fig. 11A). Other time periods of silibinin exposure also showed moderate increase, however, maximum effect was evident at 6 hrs (Fig. 11A). In case of Rb2/p130, silibinin treatment resulted in 1.4-2.3-fold increase in its hypophosphorylated form after 6 hrs of treatment, which persisted during longer treatment time of 12 and 24 hrs (Fig. 11B). Silibinin treatment, however, did not show any noticeable change in protein expression of E2F3 and E2F5 (Fig. 12). Together, these results suggest that the observed induction in hypophosphorylated levels of Rb-related proteins by silibinin could possibly be due to an upstream growth inhibitory signal that changes phosphorylation status of Rb-related proteins but is unresponsive to the expression of E2Fs. Those effects are detailed later in Task IIIc.

Negative findings: As discussed above in the studies assessing the effect of silibinin on E2F protein levels, we did not observe any change in both E2F3 and E2F5 protein levels.

Methodological problems: We have had hard time separating hypophosphorylated RB/p107 band (fast migrating) from hyperphosphorylated band (slow migrating). Similarly, we also have had hard time separating hypophosphorylated RB/p130 band (fast migrating) from hyperphosphorylated band (slow migrating). Several changes were made in the percentage of PAGE gel content ranging from 6% to 12%, and finally by using 6% gels and over running the gels, we were able to separate these bands.

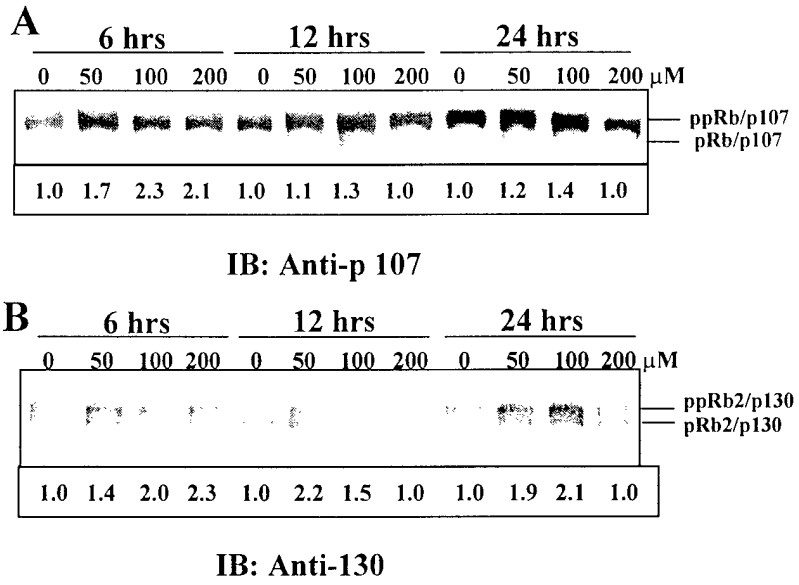

Figure 11. Silibinin induces hypophosphorylation of Rb/p107 and Rb2/p130 in human PCA DU145 cells. Cells were treated with either vehicle alone or varying concentrations of silibinin for 6, 12 and 24 hrs, total cell lysates were prepared, and subjected to SDS-PAGE on 6% gels followed by Western blotting. Membrane was probed with anti- **(A)** Rb/p107 or **(B)** Rb2/p130 antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone. ppRb/p107, hyperphosphorylated Rb/p107; pRb/p107, hypophosphorylated Rb/p107; ppRb2/p130, hyperphosphorylated Rb2/p130; pRb2/p130, hypophosphorylated Rb2/p130. IB, immunoblotting.

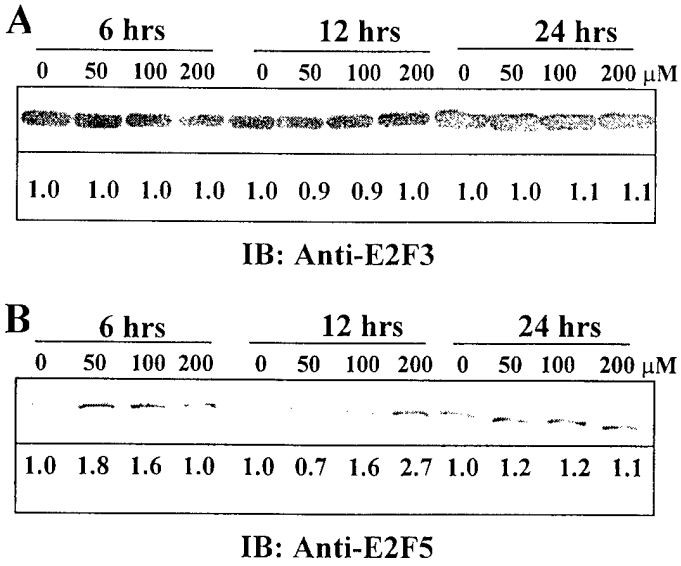

Figure 12. Lack of silibinin's effect on protein levels of E2F3 and E2F5 in human PCA DU145 cells. Cells were treated with either vehicle alone or varying concentrations of silibinin for 6, 12 and 24 hrs, total cell lysates were prepared, and subjected to SDS-PAGE on 12% gels followed by Western blotting. Membrane was probed with anti- **(A)** E2F3 or **(B)** E2F5 antibody followed by peroxidase

conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone. IB, immunoblotting.

Experiment 2: Design and Methods: We next conducted studies in LNCaP human PCA cells. For such studies, LnCaP cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicilin–streptomycin under standard culture conditions. 60% confluent cells were treated with either dimethyl sulfoxide (DMSO) alone or different doses of silibinin (50, 100, 200 μM) in DMSO for 12, 24 and 48 hrs. The final concentration of DMSO in culture medium during silibinin treatment did not exceed 0.1% and the same concentration of DMSO was present in control dishes. After 12, 24 and 8 hrs of treatment, medium was aspirated, cells were washed two times with cold PBS and cell lysates were prepared .

To study the binding of Rb and E2F1, 50μg of nuclear extract per sample were clear by protein G agarose for 1 hrs, and incubated overnight with primary anti Rb antibody plus protein G agarose. Immunocomplexes were washed three times with lysis buffer. For western immunoblotting, immunocomplexes or cell lysates (40-60 μg) of protein lysate per sample were denatured with 2X sample buffer, samples were subjected to SDS-PAGE on 8, 12 or 16 % gel, and separated proteins were transferred onto membrane by western blotting. The levels of RB, Phosphoplus Rb (Ser780, Ser795, Ser807/811, Ser249/Thr252 & Thr373), E2F1, E2F2 and E2F3 were determined using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody and proteins were detected by the ECL detection kit. We would like to highlight here that same sample lysates were also used to determine CDK2, CDK4, cyclin D1, cyclin E, Cip1/p21 and Kip1/p27 (as proposed in task IIIc) for a direct comparison and establishing a cause and effect relationship.

Data from RB protein levels, phospho-RB protein levels, different E2F protein levels and RB-E2F binding levels are summarized in following pages. As shown in Figure 13, silibinin treatments resulted in a shift of Rb to the hypophosphorylated state. The Rb in LNCaP cells was completely hypophosphorylated by 200 μM dose of silibinin at 48 hrs. Rb hypophosphorylation was both time and dose dependent. In other studies, we also observed phosphorylation status of five different Ser/Thr sites of phospho Rb after silibinin treatment in LNCaP cells. Silibinin treatment showed intense decrease in hyperphosphorylation of Rb at Serine sites (Ser780, Ser795, Ser807/811, Ser249/Thr252 but we did not find any change at the Thr 393 site. Densitometric analysis of blot showed significant percentage of decrease in all four seriene sites (Ser780- 76%, Ser795-78%, Ser807/811-96% & Ser249/Thr253-93%) at 24 hrs of 100 and 200μM silibinin (Fig.14). This decrease in phosphorylation status was dose dependent.

In the studies assessing the effect of silibinin on protein expression of E2F1, E2F2 and E2F3 in LNCaP cells, we found dramatic decrease in E2F2, E2F3 protein expression and observed moderate decrease in E2F1 protein expression (Fig. 15). Maximum decrease in protein expression of E2F2 and E2F3 was 98.02% and 88.45%, respectively (Fig. 15). In the studies evaluating the binding of E2F1, 2 and 3 with Rb in silibinin treated LNCaP cells, we observed a strong increase in the binding between Rb-E2F1 (3.8 fold) and Rb- E2F2, 3 (2.2 folds, Fig. 16) after 24 hrs of 200μM silibinin treatment.

Negative findings: We did not find any changes in E2F1 protein levels by silibinin in these studies.

Methodological problems: We did not observe any methodological problems in these studies.

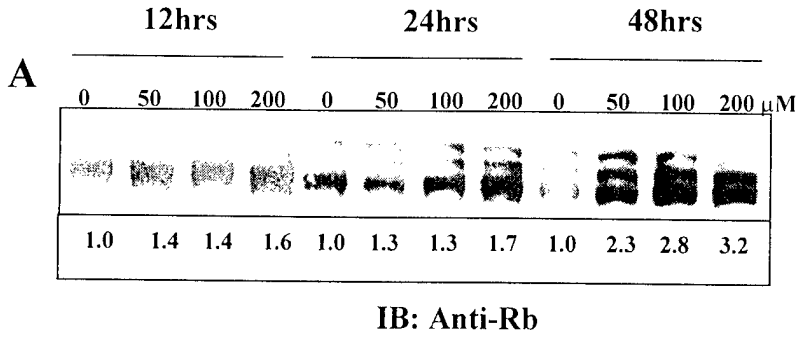

Figure 13. Silibinin induces hypophosphorylation of Rb in human PCA LNCaP cells. Cells were treated with either vehicle alone or varying concentration of silibinin for 12, 24 and 48 hrs. At the end of these treatments, total cell lysates for Rb were prepared, and subjected to SDS-PAGE on 8% gels followed by western blotting. Membrane was probed with anti-Rb antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone.

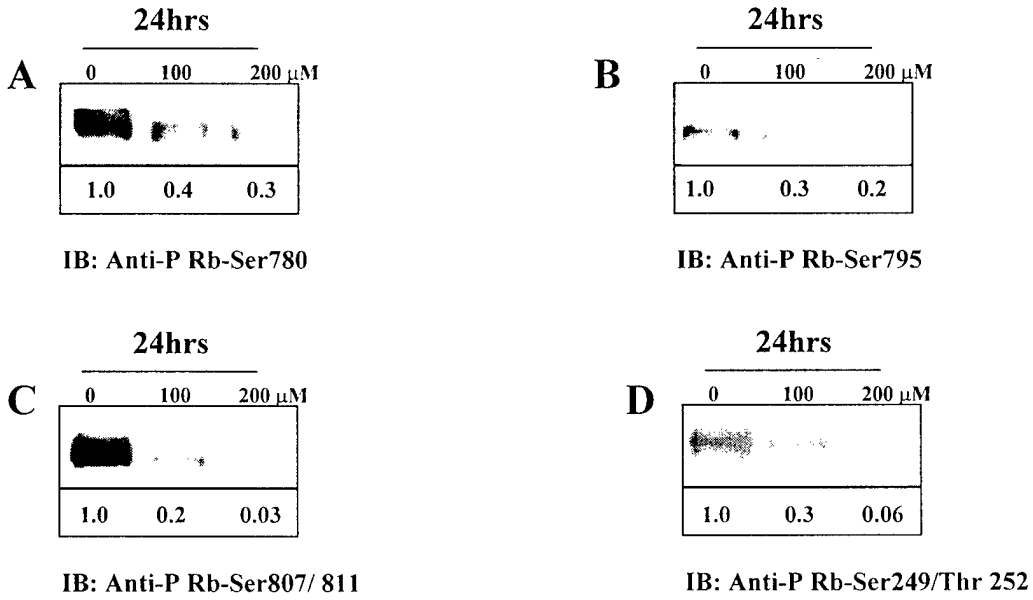

Figure 14. Silibinin induces seriene hypophosphorylation of Rb in human PCA LNCaP cells. Cells were treated as detailed above, nuclear extract prepared, and subjected to SDS-PAGE on 8% gels followed by western blotting. Membranes were probed with different phospho-specific anti-Rb

antibodies followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure.

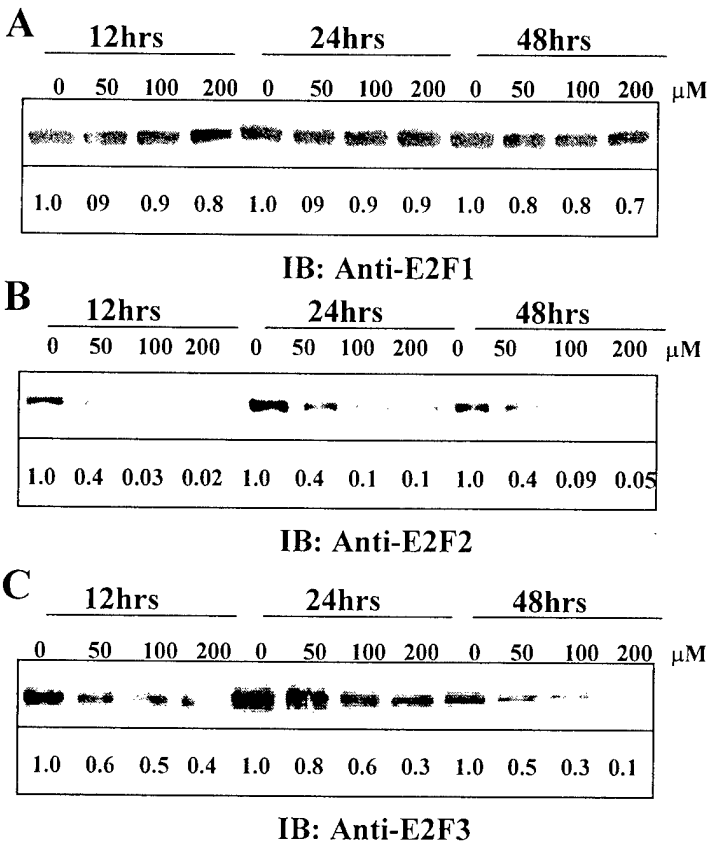

Figure 15. Silibinin modulates different E2F protein levels in human PCA LNCaP cells. Cells were treated with either vehicle alone or varying concentration of silibinin for 12, 24 and 48 hrs. At the end of these treatments, total cell lysates were prepared, and subjected to SDS-PAGE on 12% gels followed by western blotting. Membrane was probed with different E2F antibodies followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone.

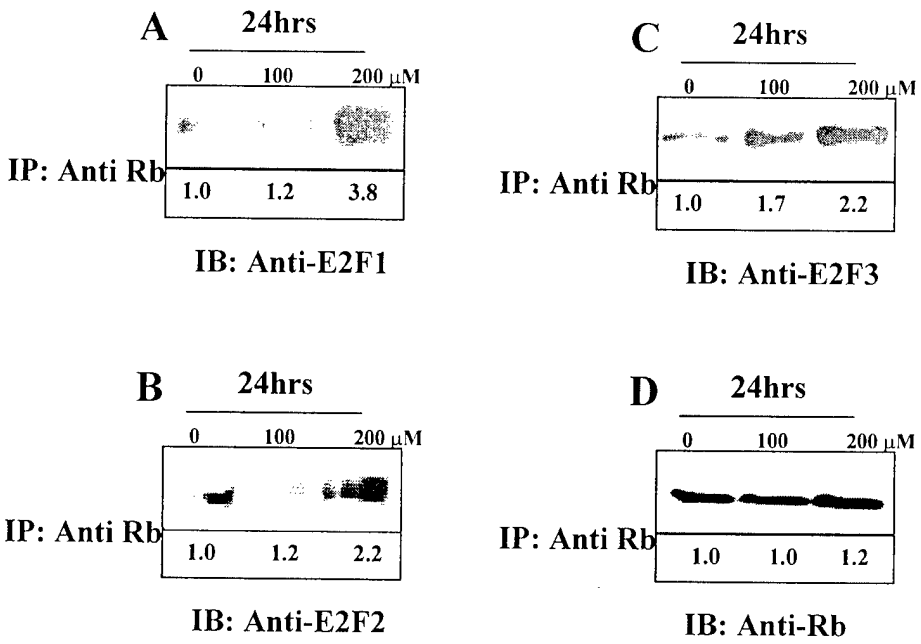

Figure 16. Silibinin modulates the binding of Rb with different E2F in human PCA LNCaP cells. Cells were treated with either vehicle alone or varying concentration of silibinin for 24 hrs. At the end of these treatments, total cell lysates were prepared, and subjected to immunoprecipitation with anti-Rb antibody. Immuno-complexes were subjected to SDS-PAGE followed by western blotting. Membrane was probed with different E2F antibodies or Rb antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone.

6.3.c Assess the effect of silymarin on CDKIs, CDKs and cyclins in LNCaP and DU145 cells: For all the studies, the pure form of silymarin, namely "silibinin" was used.

Design and Methods: Same lysates that were used for Rb, Rb-related proteins and different E2F proteins levels detailed above in section 6.3.a & b for both DU145 and LNCaP cells, were used for these studies. This helped us in identifying the up-stream targets responsible for the observed changes in phosphorylation status of Rb or related proteins by silibinin in these cells, and establishing a cause and effect relationship. Briefly, cell lysates were subjected to SDS-PAGE, and separated proteins were transferred onto membrane by

Western blotting. The levels of different CDKIs, CDKs and cyclins determined using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody and ECL detection.

As shown in Figure 17, treatment of DU145 cells with different concentrations of silibinin resulted in strong induction of Cip1/p21, Kip1/p27 and p15 protein levels. The densitometric analysis of the blots for Cip1/p21 showed that 6, 12 and 24 hrs silibinin treatments resulted in approximately 3-, 8- and 13-fold induction, respectively. Similar to its effect on Cip1/p21 induction, treatment of cells with silibinin also showed an up-regulation of Kip1/p27 protein levels. However, in this case, maximum induction (~6-fold) was evident after 12 hrs of treatment. In the studies assessing the effect of silibinin on INK family of CDKIs, p18 levels did not change following silibinin treatments at different doses and time periods. In case of p15, silibinin treatment showed up to ~2-fold induction in its protein levels following 24 hrs of treatment. In the studies assessing the effect of silibinin on the protein levels of CDKs and cyclins, it showed strong decrease in the expression of CDK4 and CDK2 levels. The densitometric analysis of the blots for CDK4 protein levels showed that compared to vehicle control, silibinin treatment for 24 hrs resulted in up to 90% decrease. In case of CDK2, however, maximum effect of silibinin was evident after 12hrs treatment accounting for up to 70% decrease. Unlike its effect on CDKIs and CDKs expression, silibinin treatment of DU145 cells did not result in any change in cyclin D1 and cyclin E levels.

Similar to its effect in DU145 cells, as shown in Figure 18, treatment of LNCaP cells with indicated concentration of silibinin showed an induction of CDKI Cip1/p21, Kip1/p27 protein expression, in both dose and time dependent. Maximum induction 2.4 fold in Cip1/p21 protein expression was observed at 48 hrs. At 24 hrs of silibinin treatment, induction was 1.8 fold. Silibinin also showed a strong induction in Kip1/p27 level. Maximum induction 3.6 fold was observed at 48 hrs treatment. Silibinin also showed strong effect on CDKs and cyclin levels. Densitometric analysis of blots of CDK2 and CDK4 protein levels showed 77.6 % and 97.4% decrease at 12 and 48 hrs, respectively. In case of cyclins, maximum down regulation of 63.3% in cyclin D1 and 29.4% in cyclinE was observed at 48 hrs silibinin treatment.

Negative findings: We did not observe any effect of silibinin on certain INK family of CDKI and cyclin D1 and E in DU145 human PCA cells.

Methodological problems: We did not encounter any methodological problems in these studies.

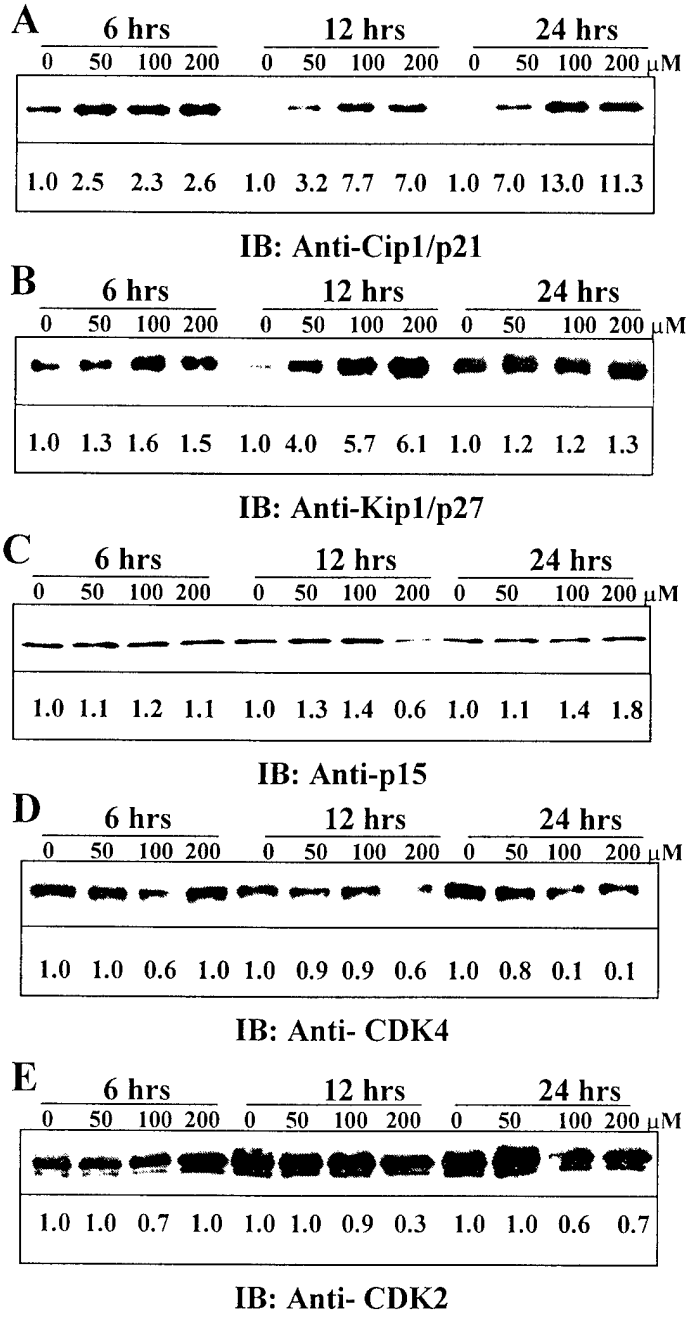

Figure 17. Silibinin modulates CDKIs and CDKs in human PCA DU145 cells. Cells were treated with either vehicle alone or varying concentration of silibinin for desired time, and total cell lysates were prepared and subjected to SDS-PAGE followed by western blotting. Membrane was probed with different antibodies followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone.

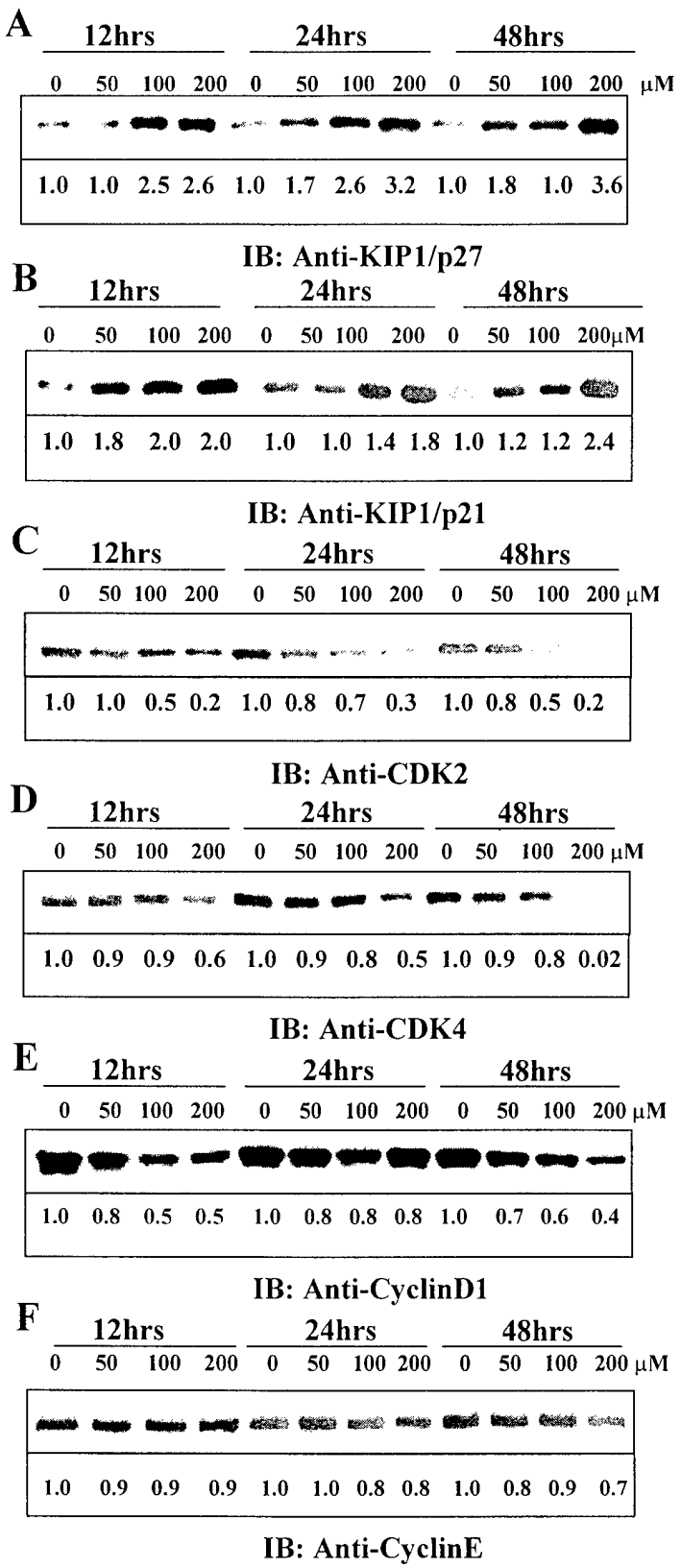

Figure 18. Silibinin modulates CDKIs, CDKs and cyclins in human PCA LNCaP cells. Cells were treated with vehicle or varying concentration of silibinin for desired time, and total cell lysates were prepared and subjected to SDS-PAGE followed by western blotting. Membrane was probed with different antibodies followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone.

6.3.d, e & f Assess the effect of silymarin on CDKI-CDK and CDK-cyclin complex formation, kinase activity of CDKs and cyclins, and cell cycle progression in LNCaP and DU145 cells: All these studies are already completed and published, and therefore are not detailed here. They are described in appendix publications #1 and #2. These studies clearly showed that silymarin or silibinin increases the binding of CDKIs to CDKs, strongly decreases kinase activity of CDKs and cyclins, and causes a strong G1 arrest in cell cycle progression of both LNCaP and DU145 human PCA cells.

6.4 Task (Aim) IV: To study the potential biological application of silymarin in the intervention of prostate cancer, Months 18-30: The outcomes of these studies are described in detail below.

In an attempt to address the biological significance of the molecular mechanistic studies described in detail above, we employed a two-tier approach. In first approach, we assessed the effect of silibinin on cell growth, DNA synthesis and biological fate (in terms of cell death), and in second attempt we focussed our efforts on nude mice PCA xenograft studies.

6.4.a Effect of silymarin on growth, DNA synthesis and ultimate biological fate of LNCaP and DU145 cells: For all the studies, the pure form of silymarin, namely "silibinin" was used. First we performed the studies to assess the effect of silibinin on LNCaP and DU145 PCA cell growth. As shown by data in **Figure 19**, treatment of LNCaP and DU145 human prostate carcinoma cells with silymarin (in its pure form, silibinin) resulted in a highly significant inhibition of cell growth in both dose- and time-dependent manner. Whereas low doses of silibinin (10 and 25 μg/ml) were not effective at all the time points studied, the doses higher than these (50, 75 and 100 μg/ml silibinin) showed strong inhibitory effect on cell growth in both the cell lines (**Figure 19**). The treatment of these cells with higher doses of silibinin for longer treatment period also resulted in a moderate cell death (**Figure 19**). The observed dose- and time-dependent inhibitory effect of silibinin on cell growth strongly corroborated with its dose- and time-dependent inhibitory effect on the molecular events detailed above suggesting that the two are causally related in inhibiting prostate cancer growth by silymarin.

We next assessed the effect of silibinin on DNA synthesis in these cells. The effect of silibinin on DNA synthesis was assessed employing bromodeoxyuridine (BrdU) incorporation using colorimetric ELISA kit (from Boehringer Mannheim Corp., Indianapolis, IN). Several different treatment protocols were used to assess the effect of silibinin on DNA synthesis in presence of serum, and serum starved conditions in the absence or presence of EGF following silibinin pre-treatment. Briefly, in first study, 1000 cells/well were cultured in 96 well plates at 37°C for 24 hrs in medium containing 10% serum, and then re-fed with fresh medium containing DMSO or 50, 75 and 100 μg/ml doses of silibinin in DMSO. After 24 hours, BrdU was added with subsequent incubation for another two to three hours at 37°C. Thereafter, DNA was denatured and cells were incubated with anti-BrdU antibody followed by addition of substrate. The reaction product was quantified by measuring absorbance at 450 nm wavelength using a scanning multi-well spectrophotometer. In other studies, 24 hrs after identical cell seeding, cells were washed with serum free medium and serum starved for 34 hrs. They were then treated with same doses of silibinin alone or silibinin followed 2 hrs later EGF (50 ng/ml). After 24 hrs, DNA synthesis was determined in both sets of treatments as detailed above.

Similar to its cell growth inhibitory effects, silibinin treatment also resulted in moderate to strong inhibition of DNA synthesis under different culture conditions. As shown in Figure 20, treatment of LNCaP and DU145 cells grown under normal serum condition with silibinin resulted in significant inhibition of DNA synthesis at all three 50, 75 and 100 μg/ml doses accounting for 35, 42 and 60% inhibition in case of LNCaP and 26, 37 and 48% inhibition in case of DU145 cells, respectively. When similar treatments of silibinin were done in serum starved cultures, the significant inhibition was also evident in both the cell lines at 50 μg/ml dose, no further inhibition, however, was evident by increasing the dose (Figure 20). Comparable to its effect on DNA synthesis in serum starved cells, 50 μg/ml dose of silibinin showed ~40% inhibition when starved cultures following silibinin treatment for 2 hrs were stimulated with EGF (Figure 20). Higher doses in this treatment protocol, however, showed additional inhibition accounting for ~50% inhibition.

Negative findings: We did not observe any negative findings in these studies.

Methodological problems: We did not encounter any methodological problems in these studies.

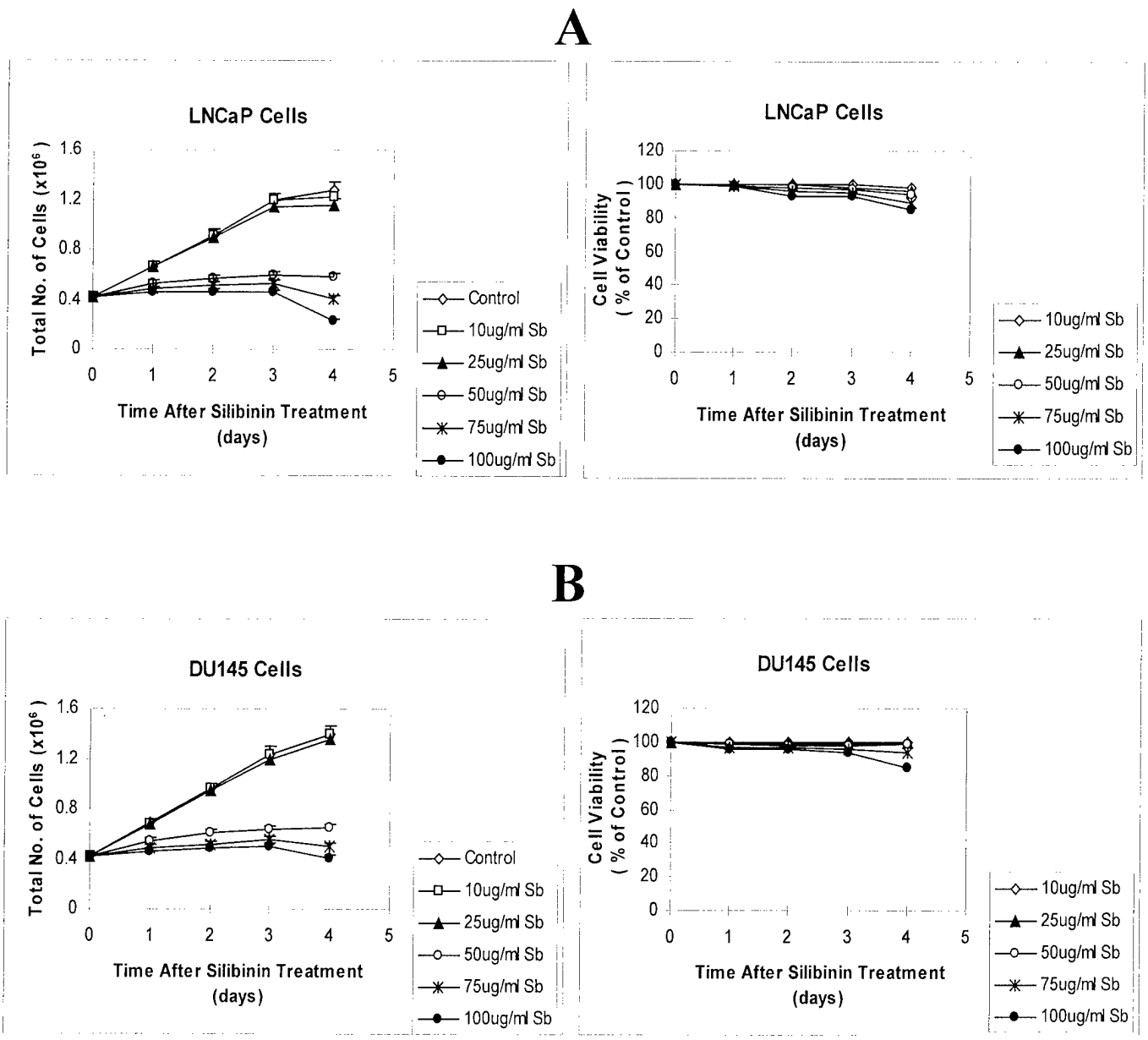

Figure 19: Inhibitory effect of silibinin on the growth of LNCaP and DU145 human prostate carcinoma cells in culture. LNCaP and DU145 cells were seeded at 4 x 105 cells/35 mm dish under standard culture conditions in RPMI 1640 medium with 10% serum and 1% P-S. After 24 hrs, the medium was removed and cultures were treated with either DMSO alone (control) or varying doses of silibinin in DMSO. The medium was changed alternate days with desired amount of fresh silibinin upto the end of the study. At 1, 2, 3 and 4 days after these treatments, cells were trypsinized and both live and dead (by trypan blue staining) cells were counted.

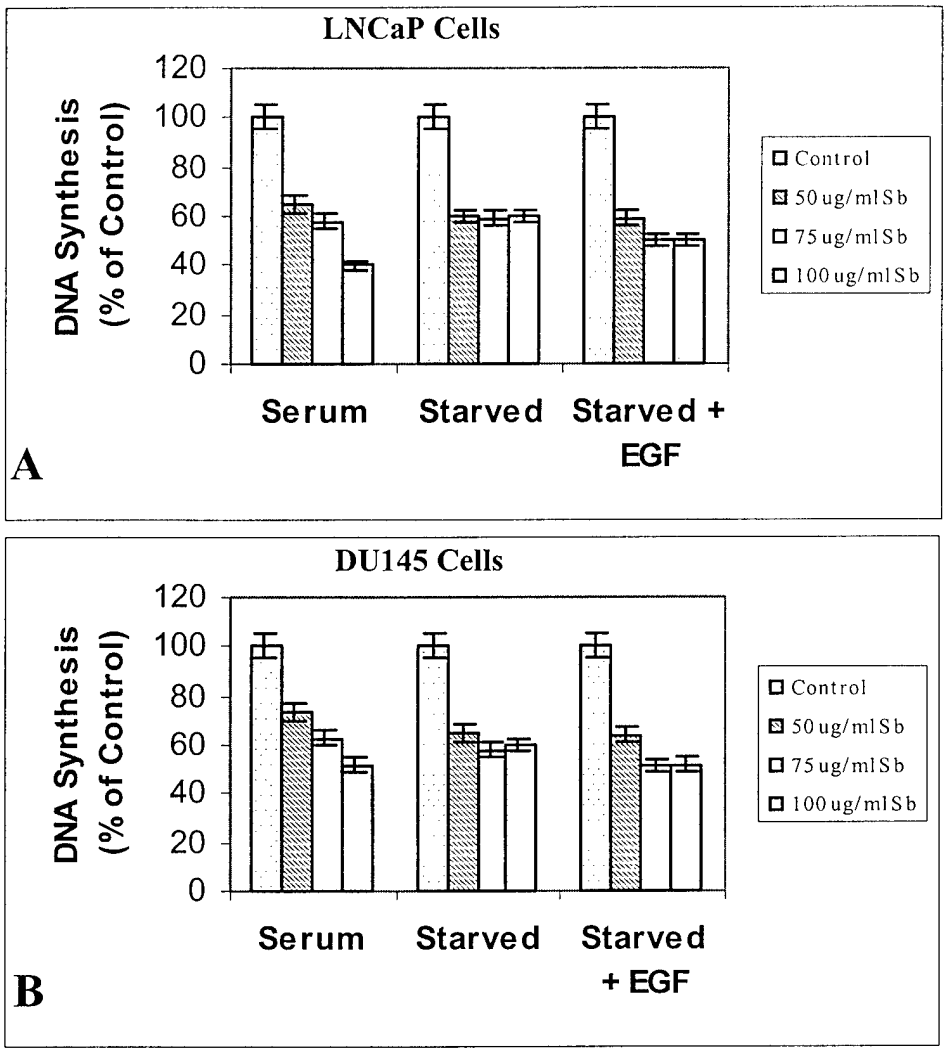

Figure 20. Effect of silibinin on DNA synthesis. LNCaP (panel A) and DU145 (panel B) cells at 1000 cells/well were cultured in 96 well plates for 24 hrs in medium containing 10% serum, and then re-fed with fresh medium containing DMSO or 50, 75 and 100 μg/ml doses of silibinin. After 24 hrs, BrdU was added with subsequent incubation for another two to three hours, DNA was denatured and cells were incubated with anti-BrdU antibody followed by addition of substrate. The reaction product was quantified by measuring absorbance at 450 nm wavelength using an ELISA reader as detailed in Methods. In other studies, 24 hrs after identical cell seeding, cells were washed with serum free medium and serum starved for 34 hrs. They were then treated with same doses of silibinin or silibinin followed 2 hrs later EGF (50 ng/ml). After 24 hrs, DNA synthesis was determined in both sets of treatments as detailed in Methods. In each case, the data shown are mean ± SD of two independent studies, each study had four independent experimental wells.

6.4.b Effect of silymarin on human PCA xenograft growth in nude mice: Several studies were done in this task to standardize human LNCaP and DU145 tumor xenografts in male nude mice, standardizing the immunohistochemical staining techniques using the tumor xenograft tissue samples from the tumor studies, and conducting pilot studies to assess the response of oral feeding of silymarin by gavage on DU145 tumor growth in male nude mice. These studies, their outcomes and methodological problems are detailed below.

6.4.c LNCaP and DU145 tumor xenograft growth in male nude mice: Male Balb/c Nu/Nu mice were purchased from NCI, MD, and the animals were maintained in Nude Mice Facility at AMC Cancer Research Center. For each cell line, mice were divided in two groups of 10 animals each. Approximately one million cells (either LNCaP or DU145) suspended in 0.25 ml of medium and mixed with 0.25 ml of Matrigel were

injected s.c. on right flanks of each mouse. The time of tumor appearance (measurable tumors) was recorded for individual mouse in each group. The tumor size was measured two times per week using vernier caliper, and the tumor volume calculated using the equation (a^2 x b)/2, a and b are width and length in mm, respectively. The experiment was terminated at day 42 from the day of initial cell inoculation. At this time, mice were sacrificed, and tumors were excised and stored appropriately for desired immunohistochemical studies. As shown by data in Figure 21, inoculation of both LNCaP and DU145 cells sub-cutaneously to male nude mice resulted in nice tumor xenograft growth in both cases. Whereas initially the xenograft growth was slow up to 21 days, they started growing aggressively thereafter.

Negative findings: We did not get any negative findings. In fact, we were able to standardize these models.

Methodological problems: Whereas we did not encounter any methodological problems, the tumor growth was kind of slow during first 21 days of inoculation. Discussing this issue with other scientist working in this area revealed that using freshly thawed PCA cells in these studies could take care of this problem.

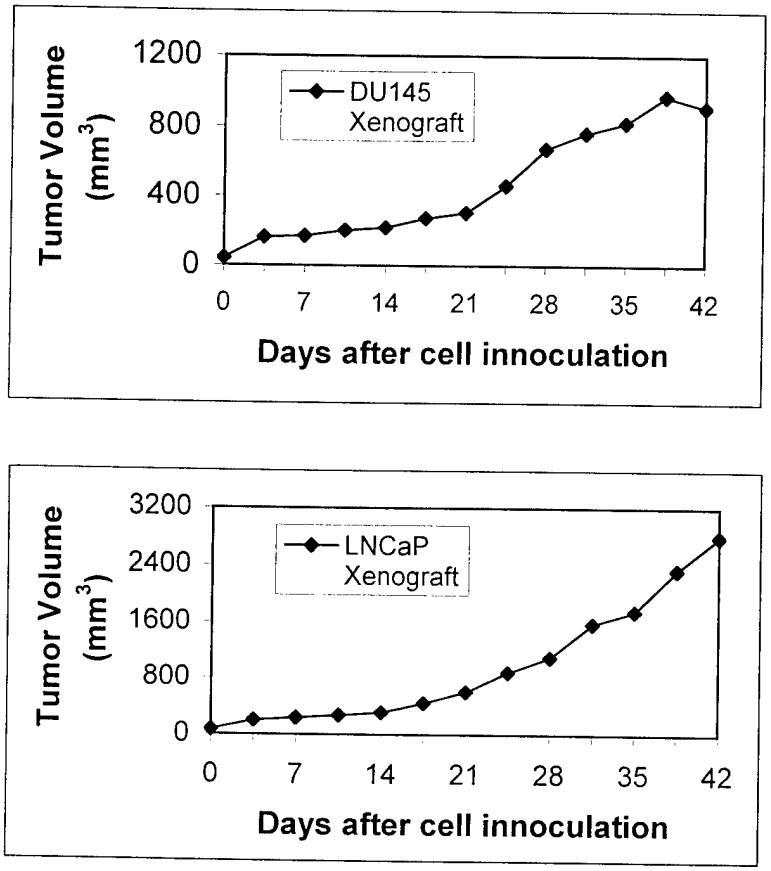

Figure 21: Human LNCaP and DU145 tumor xenograft growth in male nude mice. Experimental details are those described above.

6.4.d Immunohistochemical Detection of PCNA in Nude Mice Tumors: PCNA is a potentially useful molecular biomarker of cellullar proliferation kinetics. This fits to the expected biological mechanisms and can be correlated to the decreased/increased cancer incidence. Briefly, all the tumor samples were fixed in 10%

buffered formalin for 24 h and processed conventionally. The paraffin-embedded tumor sections (5μm thick) were heat immobilized, and deparaffinized using xylene and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was done in 10 mM citrate buffer (pH 6.0) in microwave for 2 and 18 min at full and 20% of power levels, respectively. Endogenous peroxidase activity was blocked by immersing the sections in 3.0 % H_2O_2 in methanol (v/v), followed by three changes in 10 mM PBS (pH 7.4). The sections were then incubated with mouse monoclonal anti-PCNA antibody IgG2a (Dako), 1:400 in PBS for 1 h at 37^0C in humidity chamber. Negative controls were treated only with PBS under identical conditions. The sections were then incubated with biotinylated rabbit anti-mouse antibody IgG (1:200 in 10% normal rabbit serum from Dako) for 30 min at room temperature. Thereafter, following wash with PBS, sections were incubated with conjugated horseradish peroxidase streptavidin (Dako), 1:1000 in PBS for 30 min at room temperature in humidity chamber. The sections were then incubated with 3,3'-diaminobenzidine (Sigma) working solution for 10 min at room temperature and counterstained with diluted Harris hematoxylin for 2 min, and rinsed in Scott's water. The slides were then dehydrated, mounted, viewed and photographed. Proliferating cells were quantified by counting the PCNA-positive cells and the total number of cells at 10 arbitrarily selected fields at 400-fold magnification in a double-blinded manner. The proliferation index (per 400-fold microscope field) was determined as number of PCNA-positive cells X 100 / total number of cells. Using these techniques, as shown in Figure 22, we were successful in finding a nice specific PCNA staining in both LNCaP and DU145 tumor xenografts. As shown in Figure 22 also, the proliferation index was found to be 19.8±2.5 and 7.9±1.9 in case of LNCaP and DU145 tumor xenograft tissue section staining for PCNA, respectively.

Negative findings: We did not get any negative findings. In fact, we were able to standardize PCNA staining technique.

Methodological problems: We did not encounter any methodological problems.

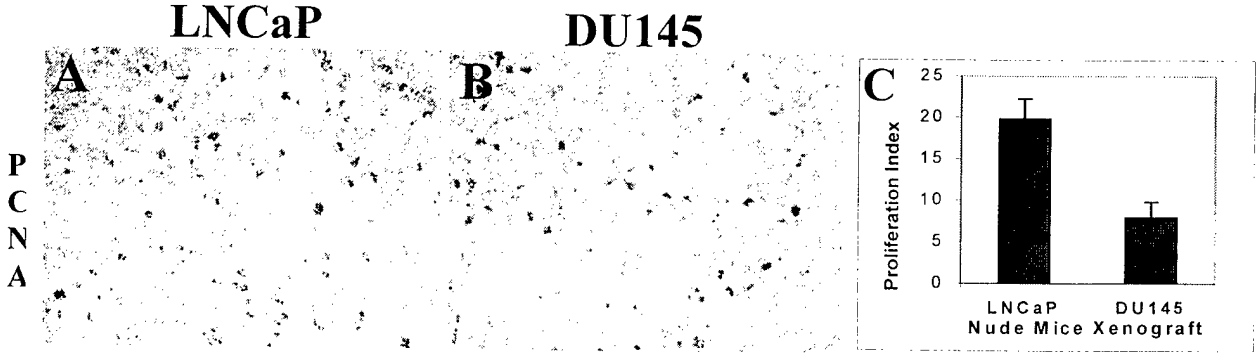

Figure 22: PCNA staining and proliferation index in LNCaP and DU145 tumor xenograft sections. The methodological details are described above.

6.4.e In-situ apoptosis detection by TUNEL staining in Nude Mice Tumors: In order to identify early as well as late apoptotic cells, all the nude mice tumors were studied by TUNEL staining. DNA fragmentation in individual apoptotic cell was visualized by detection of biotinylated nucleotides incorporated onto the free 3'-

hydroxyl residues of these DNA fragments by Tumor TACS *in situ* Apoptosis Detection Kit (R & D Systems, Inc. Minneapolis, MN). Briefly, tumor samples were first fixed in 10% buffered formalin for 8 h to prevent the loss of low molecular weight DNA fragments, then paraffin-embedded 5 μm thick sections were cleared in xylenes and rehydrated in graded concentrations of ethanol. Slides were rinsed with Ca^{++}, Mg^{++} and DNAase free phosphate buffered saline (10 mM PBS, pH 7.4) and permeabilized with proteinase K at room temperature to make the DNA accessible to the labeling enzyme. For positive control, section was incubated with TACS-nuclease for 30 min that generated DNA strand breaks in virtually every cell. Endogenous peroxidase activity was quenched using 5% H_2O_2 (in methanol, v/v) for 5 min and sections were incubated with TdT(terminal deoxynucleotidyl transferase) labeling buffer for 5 min before starting the labeling reaction. Then sections were incubated with TdT enzyme and biotinylated nucleotides (for negative control, labeling buffer was used instead of TdT enzyme) for 1 h at 37°C in humidified chamber. The reaction was stopped by adding TdT stop buffer for 5 min. Sections were incubated with streptavidin-conjugated horseradish peroxidase for 10 min. Brown color was developed with incubation in 3,3'-diaminobenzidine working solution (1:5:5000; 30% H_2O_2:DAB:1X PBS) for 7 min at room temperature. The slides were counterstained in 1% methyl green for 1 min and visualized and scored under a light microscope. The apoptosis was evaluated by counting the positive cells (brown-stained) as well as the total number of cells at 10 arbitrarily selected fields at 400-fold magnification in a double-blinded manner. The apoptotic index (per 400-fold microscope field) was calculated as number of apoptotic cells X 100 / total number of cells. Using these techniques, as shown in Figure 23, we were successful in finding specific TUNEL staining in both LNCaP and DU145 tumor xenografts. As shown in Figure 23 also, the apoptotic index was found to be very low, and was 1.02±0.09 and 1.1±0.05 in case of LNCaP and DU145 tumor xenograft tissue section staining for TUNEL, respectively.

Negative findings: We did not get any negative findings. In fact, we were able to standardize TUNEL staining technique.

Methodological problems: We did not encounter any methodological problems.

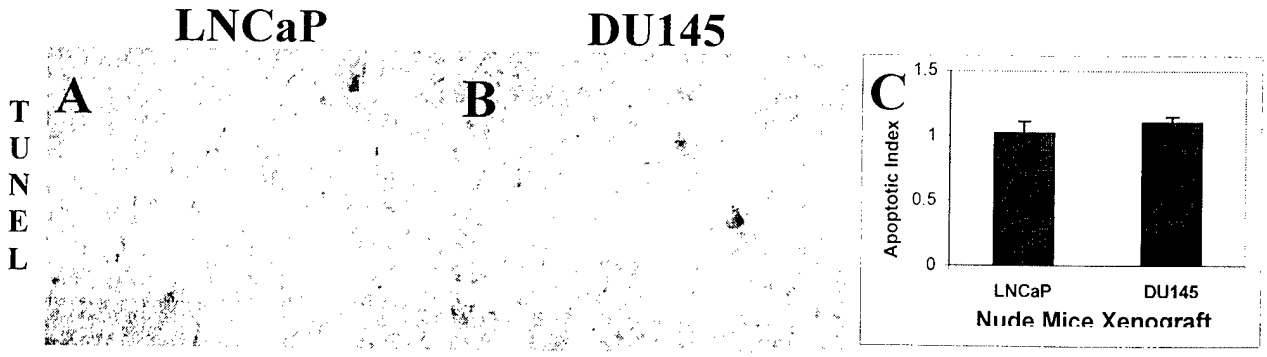

Figure 23: TUNEL staining and apoptotic index in LNCaP and DU145 tumor xenograft sections. The methodological details are described above.

6.4.f Effect of oral feeding of silymarin on DU145 tumor xenograft growth in nude mice: After standardizing the human PCA tumor xenograft models in male nude mice, and based on the mechanistic results detailed above showing strong inhibitory effects of silibinin and silymarin on different molecular pathways, next we conducted a pilot study assessing the efficacy of oral feeding of silymarin by gavage on DU145 tumor xenograft growth in nude mice. As shown in Figure 24, we found that silymarin feeding by oral gavage at 100 mg/kg dose for 34 days (5 days/week), following similar study method described in detail above for standardizing tumor xenograft protocol, results in ~80% inhibition in DU145 wet tumor xenograft weight at the end of the study in nude mice.

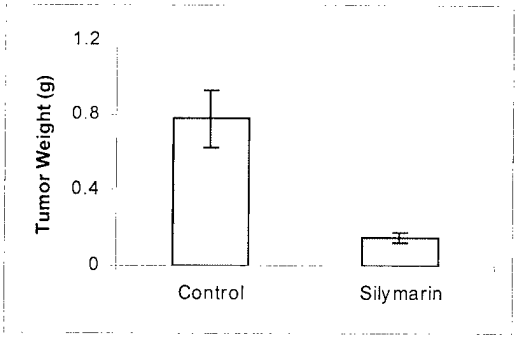

Figure 24: Inhibitory effect of oral feeding of silymarin on DU145 tumor xenograft weight in nude mice.

Negative findings: We did not get any negative findings in these studies.

Methodological problems: Unfortunately we encountered two major methodological problems in these studies. First, delivering silymarin and vehicle by oral gavage was a big problem, and in fact few mice died. Secondly, tumor volume data were inconclusive for two reasons. First, they were not consistent for the tumors on left and right sites of cell inoculation, and secondly they were responsive to silymarin feeding in some case and not in other case when compared in terms of left versus right site of xenograft.

Alternative strategies: Since we realized that this is crucial data showing ~80% inhibition in wet tumor volume by silymarin, and since we realized that the experimental approach we are using in terms of silymarin feeding and tumor cell injection on both left and right flanks is creating methodological problems, alternative strategy was made with following changes. First we rationalized that since silymarin is used as a dietary supplement, feeding it in diet may be a more practical and translation approach. Secondly, we dropped the idea of inoculating cells on both left and right flank, and decided to go only with right flank. Presently, the studies are in progress where we are using commercially prepared diet containing silymarin (as pure form namely silibinin) in diet at 0.05 and 0.1% (weight by weight of diet). In these studies, male nude mice (10/group) are being fed with control, 0.05% silibinin in diet or 0.1% silibinin in diet, ad libitum, and are being inoculated with LNCaP or DU145 cells on the right flank. We are hopeful that this alternative strategy will be useful in deriving meaning full and important information from these studies.

7. KEY RESEARCH ACCOMPLISHMENTS

There were several key research accomplishments, which include both academic and professional achievements. The **academic accomplishments** are summarized below.

- Treatment of LNCaP and DU145 human prostate carcinoma cells with silymarin results in a highly significant inhibition of TGFα binding to erbB1 receptor in both dose- and time-dependent manner.
- Consistent with above finding, silymarin also showed a strong inhibition of ligand (TGFα) internalization in these two cell lines.
- Conversely, silymarin does not result in the inhibition of intrinsic tyrosine kinase activity of erbB1 in both LNCaP and DU145 cells.
- The observed inhibitory effect of silymarin on ligand binding to erbB1 and ligand internalization also resulted in an inhibition of erbB1 activation followed by its dimerization that leads to activation of downstream mitogenic signaling.

- These inhibitory effects of silymarin on LNCaP and DU145 cells also corroborate with its inhibitory effect on both cellular and released expression of TGFα in these two cell lines.
- Together these effects of silymarin resulted in a strong inhibition of constitutive MAPK/ERK1/2 activation in both LNCaP and DU145 cells.
- Silymarin also resulted in ligand-caused activation of erbB1 and MAPK/ERK1/2 in both LNCaP and DU145 cells.
- Silymarin showed a strong switch from hyperphosphorylation of RB to hypophosphorylation of RB in LNCaP cells, and hyperphosphorylation of RB-related proteins to their hypophosphorylation in DU145 cells.
- The effect silymarin on an induction of hypophosphorylation of RB or related proteins was due to an induction of CDKIs, decrease in CDK levels and decrease in cyclin levels (only in LNCaP) in PCA cells.
- Silymarin's effect on cell cycle modulators also resulted in strong decrease in CDK and cyclin kinase activity.
- Silymarin resulted in strong G1 arrest in both LNCaP and DU145 cell cycle progression.
- Silymarin caused strong cell growth inhibition with minimal cytotoxicity in LNCaP and DU145 cells.
- We were able to standardize both LNCaP and DU145 nude mice tumor xenografts.
- We were able to standardize immunohistochemical techniques for PCNA and TUNEL staining in tumor xenografts.
- Silymarin showed ~80% inhibition of DU145 tumor xenograft wet weight in a pilot study.
- We were able to identify methodological problems in oral gavage studies, and that the studies are in progress with dietary feeding of silymarin (as silibinin) for its efficacy on both LNCaP and DU145 tumor xenografts in nude mice.
- We are also pleased to report here that through a supplemental grant to our University of Colorado Cancer Center, on complementary and alternative medicine, from NCI, we have received fundable priority to start phase I/phase II clinical trials with milk thistle extract containing ~80% silymarin in prostate cancer patients. This is another major accomplishment out of the initial funding of present grant by DOD.

In terms of **professional accomplishments**, based on the results from these studies published in two respected journals (51,54), the P.I. has been invited to present these findings at several international and national meetings. The P.I. was also able to develop his academic career in prostate cancer research that is another major accomplishment. Based on the knowledge gained by him in the area of receptor mitogenic signaling in prostate cancer, he has received a RO1 grant funding from NCI, NIH starting January 1, 2000 in the area of receptor mitogenic and anti-apoptotic signaling in prostate cancer and their impairment by phytochemicals. The P.I. was also recently offered a Professors position at University of Colorado Health Sciences Center, which is his present affiliation. These accomplishments are further highlighted (in a bullet format) in the next section.

8. REPORTABLE OUTCOMES

Manuscript:

1. Zi, X., Grasso, A.W., Kung, H.-J. and **Agarwal, R.**: A flavonoid antioxidant silymarin inhibits activation of erbB1 signaling, and induces cyclin-dependent kinase inhibitors, G1 arrest and anti-carcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res., 58: 1920-1929, 1998. (**Appendix 1**).
2. Zi, X. and **Agarwal, R.**: Silibinin decreases prostate-specific antigen with cell growth inhibition via G1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention. Proc. Natl. Acad. Sci. USA, 96:7490-7495, 1999. (**Appendix 2**).
3. Agarwal, C., Sharma, Y. and **Agarwal, R.**: Anticarcinogenic effect of a polyphenolic fraction isolated from grape seeds in human prostate carcinoma DU145 cells: modulation of mitogenic signaling and cell cycle regulators and induction of G1 arrest and apoptosis. Mol. Carcinogenesis, 28: 129-138, 2000. (**Appendix 3**).
4. Jiang, C., **Agarwal, R.** and Lu, J.: Anti-angiogenic potential of silymarin: inhibition of key attributes of vascular endothelial cells and angiogenic cytokine secretion by cancer epithelial cells. Biochem. Biophys. Res. Commun., 276: 371-378, 2000. (**Appendix 4**).

5. **Agarwal, R.**: Cell signaling and regulators of cell cycle as molecular targets for prostate cancer prevention by dietary agents. Biochemical Pharmacol., 60: 1051-1059, 2000. (**Appendix 5**).
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7. Bhatia, N. and **Agarwal, R.**: Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on epigenetic events in human prostate carcinoma DU145 cells. Prostate, 46: 98-107, 2001. (**Appendix 7**).
8. Sharma, Y., Agarwal, C., Singh, A.K. and **Agarwal, R.**: Inhibitory effect of silibinin on ligand binding to erbB1 and associated mitogenic signaling, growth, and DNA synthesis in advanced human prostate carcinoma cells. Mol. Carcinogenesis, 30: In Press, 2001. (**Appendix 8**).
9. Tyagi, A., Agarwal, C. and **Agarwal, R.**: Silibinin causes hypophosphorylation of Rb/p107 and Rb2/p130 via induction of CDKis and decrease in CDKs leading to G1 arrest, growth inhibition and differentiation of human prostate carcinoma DU145 cells. In Preparation. (**Appendix 9**).

Abstracts and Presentations

10. **Agarwal, R.**: Prostate Cancer Prevention by an antioxidant silymarin. **Invited Talk** at International Conference on Diet and Prevention of Cancer, Tampere, Finland, June, 1999.
11. **Agarwal, R.**: Mitogenic and anti-apoptotic signaling as molecular targets for prostate cancer prevention by an antioxidant silymarin. **Invited Talk** to be delivered at Endocrinology Seminar at University of Colorado Cancer Center, December, 1999.
12. Zi, X. and **Agarwal, R.**: Ligand-induced receptor tyrosine kinase endocytosis in prostate cancer cells. Proc. Am. Assoc. Cancer Res., 40:614, 1999.
13. Zi, X. and **Agarwal, R.**: Impairment of erbB1-mediated endocytosis signaling by phytic acid: a novel strategy for the secondary prevention of prostate cancer. Proc. Am. Assoc. Cancer Res., 40:654, 1999.
14. **Agarwal, R.**: Cell signaling, regulators of cell cycle and apoptosis as molecular targets for prostate cancer intervention by dietary agents. **Invited Talk** to be delivered at Third Molecular and Cellular Biology Meeting, Luxembourg, January 26-29, 2000.
15. Sharma, Y, and **Agarwal, R.**: A flavonoid antioxidant silibinin inhibits TGFa expression and it's binding to erbB1 resulting in impairment of ligand/receptor autocrine growth loop in human prostate carcinoma cells. Abstract submitted for presentation in Annual Meeting of the AACR, 2000.
16. Jiang, C., **Agarwal, R.** and Lu, J.: Silymarin, a flavonoid phytochemical, inhibts key angiogenic attributes of vascular endothelial cells. Proc. Am. Assoc. Cancer Res., 41:847, 2000.
17. Bhatia, N., Sharma, Y., Zhao, J. and **Agarwal, R.**: Therapeutic efficacy of a cancer preventive agent, silymarin: involvement of anti-proliferative and cell deletion effects via inhibition of mitogenic signaling. Proc. Am. Assoc. Cancer Res., 41:663, 2000.
18. Singh, R.P., Agarwal, C. and **Agarwal, R.**: Novel anti-carcinogenic function of inositol hexaphosphate in human prostate carcinoma DU145 cells: modulation of cell-cycle regulators, growth inhibition, and induction of G1 arrest. Proc. Am. Assoc. Cancer Res., 42:461, 2001.
19. Tyagi, A., Agarwal, C. and **Agarwal, R.**: Silibinin induces growth inhibition and G1 arrest via induction of CDKIs and decrease in CDKs leading to hypophosphorylation of Rb/p107 in prostate carcinoma DU145 cells. Proc. Am. Assoc. Cancer Res., 42:462, 2001.
20. Tyagi, A., Agarwal, C., Chan, D.C. and **Agarwal, R.**: Strong synergistic efficacy of silibinin and doxorubicin combination against human prostate carcinoma DU145 cells. Proc. Am. Assoc. Cancer Res., 42:462, 2001.

Patents and licenses applied for and/or issued: None.
Degrees obtained that are supported by this award: Not applicable.
Development of cell lines, tissue or serum repositories: None.
Informatics such as databases and animal models, etc: None.
Funding: The P.I. has received a RO1 grant from NCI, NIH entitled "Receptor Signaling, Phytic Acid and Prostate Cancer" Amount of funding expected: $539,598; duration of the project: 1/1/00 to 12/31/03.

Employment/research opportunities: The P.I. was first promoted from his current position (Scientist) to Senior Scientist effective 7/1/00. Recently he was offered a Professor position at University of Colorado Health Sciences Center. Currently, he is employed at this position and institution.

Research Personnel received pay from the research effort: Rajesh Agarwal, Ph.D.; Yogesh Sharma, Ph.D.; Neehar Bhatia, Ph.D.; Alpana Tyagi, Ph.D.; Anil Tyagi, Ph.D.; Sivanandhana Dhanalakshmi.

9. CONCLUSIONS

9.1 Summary of results from completed studies including their importance and/or implications: The findings obtained from the studies detailed above in section 6, clearly and convincingly suggest that silymarin (or silibinin) has exceptionally strong inhibitory effect on erbB1-mediated mitogenic signaling in case of both androgen-dependent and androgen-independent human prostate carcinoma cells. These effects of silymarin (or silibinin) cause impairment of MAPK/ERK1/2 mediated mitogenic signaling in both LNCaP and DU145 cells. Partially through this inhibitory mechanism and partially through an independent pathway silymarin (or silibinin) induces CDKIs, decreases CDK and cyclin expression and their kinase activity. These effects cause cell growth inhibition via a G1 arrest in cell cycle progression of both LNCaP and DU145 cells, but are not cytototxic. Pilot data also show strong possibility for tumor xenograft growth inhibition by silymarin. These results are major accomplishments towards the notion that more detailed mechanistic and tumor studies are needed to assess both preventive and interventive effects of silymarin (or silibinin) against human prostate cancer.

The **major implication** of these findings is that this effect of silymarin could be exploited to inhibit the growth of those prostate carcinomas where an interaction of autocrine/paracrine growth factors and their receptors plays a causal role in malignant cell growth and metastasis. Since advanced and androgen-independent prostate cancer growth causally depends on such interactions where an autocrine loop exists between ligand and receptor and that cell cycle progression is modulated because of alterations in the cell cycle regulators, our findings showing an inhibitory effect of silymarin on these molecular events are extremely important in developing interventive strategies against prostate cancer by silymarin.

9.2 "So what section": From the findings detailed above, it can be argued that silymarin exerts its inhibitory effect on prostate carcinoma cells by different mechanisms that are possibly linked to each other by a cause and effect relationship. For example, it can be argued that as an initial step, silymarin inhibits the binding of the ligand to erbB1 that results in an inhibition of ligand internalization. Since these steps are essential for erbB1 activation, their inhibition results in an inhibition of erbB1 activation followed by a lack of its dimerization that ultimately causes a decrease in the activation of MAPK/ERK1/2. As activation of MAPK/ERK leads to activation of transcription factor for cell growth and proliferation, its inhibition would be anticipated to lead in an inhibition of trascriptional followed by translational effects including a decrease in TGFα expression followed by its release, as observed by us in silymarin treated cells. By an independent as well as partially dependent mechanism, silymarin modulates cell cycle regulators causing G1 arrest and PCA cell growth inhibition. An important question from these arguments is **so what** if silymarin inhibits these molecular events in prostate carcinoma cells, and **so what** if these studies establish a cause and effect relationship. What is the biological significance of these findings in terms of prostate carcinoma growth? This issue was identified to be extremely important to establish the implications of the findings observed thus far. In fact, studies are in progress to demonstrate the efficacy of silibinin feeding in diet on both LNCaP and DU145 tumor xenograft growth in nude mice. We are also pleased to report here that through a supplemental grant to our University of Colorado Cancer Center, on complementary and alternative medicine, from NCI, we have received fundable priority to start phase I/phase II clinical trials with milk thistle extract containing ~80% silymarin in prostate cancer patients. This is another major accomplishment out of the initial funding of present grant by DOD.

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11. APPENDICES

1. Zi, X., Grasso, A.W., Kung, H.-J. and **Agarwal, R.**: A flavonoid antioxidant silymarin inhibits activation of erbB1 signaling, and induces cyclin-dependent kinase inhibitors, G1 arrest and anti-carcinogenic effects in human prostate carcinoma DU145 cells. Cancer Res., 58: 1920-1929, 1998. (**Appendix 1**).
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Appendix 1

[CANCER RESEARCH 58, 1920–1929, May 1, 1998]

A Flavonoid Antioxidant, Silymarin, Inhibits Activation of erbB1 Signaling and Induces Cyclin-dependent Kinase Inhibitors, G_1 Arrest, and Anticarcinogenic Effects in Human Prostate Carcinoma DU145 Cells[1]

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ABSTRACT

Prostate cancer (PCA) is the most common nonskin malignancy and the second leading cause of cancer deaths in United States males. One practical and translational approach to control PCA is to define a mechanism-based anticarcinogenic agent(s). Recently, we showed that silymarin, a flavonoid antioxidant isolated from milk thistle, possesses exceptionally high to complete protective effects against experimentally induced tumorigenesis. Because the epidermal growth factor receptor (erbB1) and other members of the erbB family have been shown to play important roles in human PCA, efforts should be directed to identify inhibitors of this pathway for PCA intervention. In this study, we assessed whether silymarin inhibits erbB1 activation and associated downstream events and modulates cell cycle regulatory proteins and progression, leading to growth inhibition of human prostate carcinoma DU145 cells. Treatment of serum-starved cells with silymarin resulted in a significant inhibition of transforming growth factor α-mediated activation of erbB1 but no change in its protein levels. Silymarin treatment of cells also resulted in a significant decrease in tyrosine phosphorylation of an immediate downstream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. In the studies analyzing cell cycle regulatory molecules, silymarin treatment of cells also resulted in a significant induction of cyclin-dependent kinase inhibitors (CDKIs) Cip1/p21 and Kip1/p27, concomitant with a significant decrease in CDK4 expression, but no change in the levels of CDK2 and CDK6 and their associated cyclins E and D1, respectively. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs, together with a marked decrease in the kinase activity of CDKs and associated cyclins. In additional studies, treatment of cells grown in 10% serum with anti-epidermal growth factor receptor monoclonal antibody clone 225 or different doses of silymarin also resulted in significant inhibition of constitutive tyrosine phosphorylation of both erbB1 and SHC but no change in their protein levels. Furthermore, whereas silymarin treatment resulted in a significant increase in the protein levels of both Cip1/p21 and Kip1/p27, monoclonal antibody 225 showed an increase only in Kip1/p27. These findings suggest that silymarin also inhibits constitutive activation of erbB1 and that the observed effect of silymarin on an increase in CDKI protein levels is mediated via inhibition of erbB1 activation only in the case of Kip1/p27; however, additional pathways independent of inhibition of erbB1 activation are possibly responsible for the silymarin-caused increase in Cip1/p21 in DU145 cells. In other studies, silymarin treatment also induced a G_1 arrest in the cell cycle progression of DU145 cells and resulted in a highly significant to complete inhibition of both anchorage-dependent and anchorage-independent growth of DU145 cells in a dose- and time-dependent manner. Taken together, these results suggest that silymarin may exert a strong anticarcinogenic effect against PCA and that this effect is likely to involve impairment of erbB1-SHC-mediated signaling pathway, induction of CDKIs, and a resultant G_1 arrest.

INTRODUCTION

PCA[3] is the second most common malignancy (after nonmelanoma skin cancers) in American men and is the second leading cause (after lung cancer) of cancer deaths (1, 2). Statistical predictions for 1997 show that 334,500 new PCA cases will have been diagnosed, and an estimated 41,800 deaths due to this disease will have occurred in the United States alone (3). The induction of human PCA has been viewed as a multistage process, involving progression from small, latent carcinomas of low histological grade to large, metastatic carcinomas of higher grade (2). It is becoming clear that in the genesis of PCA, a variety of pathogenic pathways exist. Among the widely accepted risk factors for PCA are age, race, ethnicity, dietary habits, and androgen secretion and metabolism (4). African Americans have the highest PCA rate in the world, followed by Caucasian Americans and then Southeast Asians (1–3). Both epidemiology and laboratory studies have suggested that diet and androgen can alter PCA risk *via* a common etiological pathway (4–7).

To design and conduct mechanism-based early phase preventive intervention clinical trials, a major goal of PCA research in recent years has been to focus on the biology of normal prostate and elucidate the molecular mechanism(s) of PCA induction. Indeed, several genetic alterations have been identified that lead to the induction and/or development of human PCA (Ref. 8 and references therein). It has been shown that the early components of signal transduction pathways, specifically those of tyrosine kinases, are of utmost significance for controlled cell growth and differentiation (Ref. 9 and references therein). Ironically, a single genetic alteration in any of the cell signaling components can result in a continuous signaling, leading to an uncontrolled cell growth and proliferation. RTKs participate in transmembrane signaling, whereas non-RTKs take part in intracellular signal transduction, including signaling to the nucleus (9). Enhanced tyrosine kinase activity due to overexpression of RTKs and/or non-RTKs can lead to persistent autocrine stimulation of cells by secreted growth factors, which in turn can lead to a disease (9). Enhanced activity of tyrosine kinases has been implicated in a wide variety of human malignancies; several studies have shown increased expression of erbB family of RTKs in human malignancies, suggesting their role in the causation of this disease (8–10). With regard to human PCA, the aberrant expression of the erbB family of RTKs, such as EGFR (also known as erbB1), erbB2, and erbB3, has been demonstrated with strikingly high frequency in prostatic intraepithelial neoplasia and in invasive PCA, both primary and metastatic (8, 10–14).

In addition, EGF, TGF-α, and erbB1 have been shown to be

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[3] The abbreviations used are: PCA, prostate cancer; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; mAb 225, anti-EGFR monoclonal antibody clone 225; RB, retinoblastoma; GST, glutathione *S*-transferase; RTK, receptor tyrosine kinase; TGF-α, transforming growth factor α.

associated with the regulation of prostatic cell mitogenesis (15). For example, hormone-independent prostate carcinoma cells commonly express high levels of erbB1 and TGF-α, thus making a functional autocrine loop for the hormone-independent growth of PCA (16, 17). Using hormone-independent prostate carcinoma cell lines PC-3 and DU145, it has been shown that high-affinity, ligand-blocking monoclonal antibodies to erbB1 prevent its activation and also result in the growth inhibition of these cells (16–18). Together, these studies implicate that the erbB family of RTK-mediated signaling pathways may be contributory mechanisms for human PCA (14, 19), and therefore, one practical and translational approach for the intervention of PCA could be to identify the inhibitors of erbB family of RTK-mediated signaling pathway(s).

Several epidemiological studies, supported by long-term animal tumor experiments or *vice versa*, have suggested that microchemicals present in our diet, as well as several herbs and plants with diversified pharmacological properties, could be the most desirable agents for the prevention and/or intervention of human cancer incidence and mortality due to stomach, colon, breast, esophagus, lung, bladder, and even PCAs (20–25). Measuring the effects of these agents in cancer chemopreventive intervention studies in human populations has now become one important objective of experimental cancer research. The potential for inhibiting tumor development in both targeted high-risk and general population has increased significantly in recent years (20–25). Accordingly, many new classes of chemical compounds are being evaluated in clinical trials as cancer preventive and/or therapeutic agents for several malignancies (20–25); fewer efforts, however, have been made with regard to PCA (Refs. 26–29 and references therein). At present, about 30 classes of chemicals with such effects have been described, which may have practical implications in reducing human cancer incidence (22–28). Among these, polyphenolic antioxidants are receiving increased attention (22–25, 30).

Silymarin, a polyphenolic flavonoid antioxidant isolated from milk thistle (*Silybum marianum* (L.) Gaertn; Ref. 31), is being used clinically in Europe and Asia for the treatment of alcoholic liver diseases (32, 33). As a therapeutic agent, silymarin is well tolerated and largely free of adverse effects (Refs. 34 and 35 and references therein), so much so that it is also being marketed recently in the United States and Europe as nutritional supplement by Pure Encapsulations (Sudbury, MA). Studies on mice, rats, rabbits, and dogs, using different modes of administration, showed that silymarin is nontoxic in acute tests even at large doses (36). Similarly, it is nontoxic in subchronic and chronic tests and does not show any side effects (36); there is no known LD_{50} for silymarin in laboratory animals (35–38). Several studies have shown that silymarin affords protection against lipid peroxidation induced by xenobiotic agents (39, 40) and that it is a strong antioxidant capable of scavenging free radicals (Refs. 41–44 and references therein). In studies using the mouse skin models of carcinogenesis, we have shown that silymarin inhibits skin tumor promoter-caused induction of epidermal ornithine decarboxylase activity and mRNA expression (45) and that it possesses exceptionally high to complete protective effects against experimental tumorigenesis (46, 47). Likewise, experiments involving a mammary gland culture initiation-promotion protocol also demonstrated the ability of silymarin to inhibit tumor promotion (48). Taken together, these findings suggested a possibility that silymarin could also be a useful anticarcinogenic agent for PCA. In this study, we demonstrate the inhibitory effect of silymarin on activation of erbB1 signaling pathway in hormone-independent human prostate carcinoma DU145 cells. Furthermore, we found that inhibition of erbB1 activation by silymarin was associated with alterations in the levels of cell cycle regulatory molecules, cessation of cell cycle progression, and inhibition of both anchorage-dependent and anchorage-independent growth of DU145 cells.

MATERIALS AND METHODS

Materials. Human prostate carcinoma cell line DU145 was from American Type Culture Collection (Bethesda, MD). RPMI 1640, human recombinant TGF-α, and all other culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Silymarin was from Aldrich Chemical Co. (Milwaukee, WI). Anti-EGFR, anti-SHC, and antiphosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-Cip1/p21 antibody was from Calbiochem (Cambridge, MA). Anti-Kip1/p27 and anti-CDK4 antibodies and mAb 225 were from Neomarkers, Inc. (Fremont, CA). Antibodies to cyclin D1, cyclin E, CDK2, and CDK6; rabbit antimouse immunoglobulin- and goat antirabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibodies; and RB-GST fusion protein were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Histone H1 was from Boehringer Mannheim Corp. (Indianapolis, IN). [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was from New England Nuclear (Boston, MA). ECL detection system was from Amersham Corp. (Arlington Heights, IL).

Cell Culture Conditions and Silymarin Treatment. DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions. For studies assessing the effect of silymarin on activation of erbB1 signaling, 70–80% confluent cultures were washed twice with PBS, and then starved in serum-free medium for 36 h. After 20 h of starvation, the medium was replaced with fresh serum-free medium to remove any autocrinely secreted TGF-α ligand. During the last 2 h of starvation, the cultures were treated with either ethanol alone or varying concentrations of silymarin (25–150 μg/ml of medium) in ethanol. The final concentration of ethanol in culture medium during silymarin treatment did not exceed 0.5% (v/v), and therefore, the same concentration of ethanol was present in control dishes. At the end of these treatments, cultures were added with either PBS alone or TGF-α (50 ng/ml of medium) and incubated for 15 min at 37°C. Thereafter, medium was aspirated, and monolayers were quickly washed twice with cold PBS and added with 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, and 0.2 units/ml aprotinin) per plate. After 15 min in lysis buffer at 4°C, the cell lysate was scraped from the plate, collected in microcentrifuge tubes, and left on ice for an additional 15 min. For studies assessing the effect of silymarin on constitutive activation of erbB1 signaling, 70–80% confluent cultures grown in 10% serum were treated with either solvent alone, mAb 225 (6 μg/ml, equivalent to 40 nM), or varying concentrations of silymarin (50–100 μg/ml) for 16 h, and the cell lysates were prepared as detailed above. The lysates were cleared by centrifugation for 5 min in a tabletop centrifuge at 4°C, the supernatants were collected, and protein concentration was determined. For all other studies, DU145 cells were cultured as detail above, and 70–80% confluent cultures (without serum starvation) were treated with either ethanol alone, mAb 225 (6 μg/ml), or varying concentrations of silymarin (25–75 μg/ml of medium) in ethanol. Sixteen h after these treatments, the medium was aspirated, monolayers were quickly washed twice with cold PBS, and cell lysates were prepared as detailed above. For those studies assessing the time-dependent effect of silymarin, cultures were treated with silymarin at a concentration of 75 μg/ml of medium; cells were harvested 8, 24, and 48 h later; and lysates were prepared.

Immunoprecipitation and Western Blotting. For erbB1 signaling studies, 400 μg of protein lysate per sample were diluted to 1 ml with lysis buffer and added with 2 μg of anti-EGFR or anti-SHC antibody followed by rotating this mixture at 4°C for 4 h. Thereafter, 25 μl of protein A-agarose beads were added, and this mixture was incubated overnight at 4°C. The next day, beads were collected by centrifugation and washed four times with lysis buffer, and the immunoprecipitated erbB1 or SHC was denatured with 30 μl of 1× SDS-PAGE sample buffer. The samples were subjected to SDS-PAGE on an 8% gel, and separated proteins were transferred on to nitrocellulose membrane by Western blotting. As needed, membranes were probed with antiphosphotyrosine, anti-EGFR, and anti-SHC antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. For cell cycle regulatory molecules, 40–100 μg of protein lysate per

sample were denatured with 2 × SDS-PAGE sample buffer, samples were subjected to SDS-PAGE on 12% gel, and separated proteins were transferred on to membrane by Western blotting. The levels of Cip1/p21, Kip1/p27, CDK2, CDK4, CDK6, cyclin D1, and cyclin E were determined using specific primary antibodies followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. For studies evaluating the binding of CDKIs with CDKs, 50 μg of protein lysate per sample were mixed with 0.5 μg of anti-Cip1/p21 or anti-Kip1/p27 antibody and 5 μl of protein G-agarose beads for immunoprecipitations as described above. The immunoprecipitated proteins were denatured with sample buffer and subjected to 12% SDS-PAGE followed by Western blotting. The levels of CDKs bound to CDKIs were determined by specific primary antibodies to CDK2, CDK4, and CDK6 followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system.

Kinase Assays. CDK2- and cyclin E-associated H1 histone kinase activity was determined as described by Wu *et al.* (49). Briefly, using anti-CDK2 or anti-cyclin E antibody (2 μg) and protein A-agarose beads (20 μl), CDK2 and cyclin E, respectively, were immunoprecipitated from 200 μg of protein lysate per sample as detailed above. Beads were washed three times with lysis buffer and then once with kinase assay buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM DTT). Phosphorylation of histone H1 was measured by incubating the beads with 40 μl of "hot" kinase solution [0.25 μl (2.5 μg) of histone H1, 0.5 μl of [γ-^{32}P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography. Similarly, CDK4-, CDK6-, and cyclin D1-associated RB kinase activity was determined as described by Wu *et al.* (49) and detailed above with some modifications. Briefly, vehicle- or silymarin-treated DU145 cells were lysed in RB lysis buffer (50 mM HEPES-KOH, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 80 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin), and using anti-CDK4, anti-CDK6, or anti-cyclin D1 antibody (2 μg) and protein G-agarose or protein A-agarose beads (20 μl), specific proteins were immunoprecipitated from 200 μg of protein lysate per sample as detailed above. Beads were washed three times with RB lysis buffer and then once with RB kinase assay buffer (50 mM HEPES-KOH, pH 7.5, containing 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, and 1 mM DTT). Phosphorylation of RB was measured by incubating the beads with 40 μl of hot RB kinase solution [0.25 μl (2 μg) of RB-GST fusion protein, 0.5 μl of [γ-^{32}P]ATP, 0.5 μl of 0.1 mM ATP, and 38.75 μl of RB kinase buffer] for 30 min at 37°C. The reaction was stopped by boiling the samples in SDS sample buffer for 5 min. The samples were analyzed by 12% SDS-PAGE, and the gel was dried and subjected to autoradiography.

FACS Analysis and Cell Growth Assay. DU145 cells at 70–80% confluency were treated with either ethanol alone or silymarin at a dose of 75 μg/ml of medium in ethanol. Twenty-four and 48 h after these treatments, the medium was aspirated, monolayers were quickly washed two times with cold PBS, cells were trypsinized, and cell pellets were collected. The cell pellets were washed twice with PBS, and the cells were fixed in cold methanol and rewashed with PBS to remove methanol. After being suspended in 500 μl of PBS, cells were digested with 20 μg/ml RNase at 37°C for 30 min and chilled on ice for 10 min, and then cellular DNA was stained with propidium iodide (50 μg/ml) by incubation for 1 h at room temperature in the dark. Cell cycle distribution was analyzed by flow cytometry using the Becton Dickinson FACS system.

For cell growth assays, DU145 cells were plated at a density of 0.5×10^5 cells per 60-mm plate. On day 2, cells were fed with fresh medium and left untreated, or they were treated with ethanol alone or silymarin at doses of 5, 10, 25, 50, 75, and 100 μg/ml of medium dissolved in ethanol. In other studies, DU145 cells were plated at a density of 0.5×10^5 cells per 60-mm plate. The next day, cells were fed with fresh medium and treated with ethanol alone, mAb 225 at doses of 6 and 12 μg/ml of medium, or silymarin at doses of 50, 75, and 100 μg/ml of medium dissolved in ethanol. The cultures were fed with fresh medium with or without same concentrations of mAb 225 or silymarin every other day until the end of the experiment. Each treatment and time point had four plates. At days 1–6 after these treatments, cells were trypsinized and collected in counting vials. Each plate was washed thoroughly with isotonic buffer with 0.1% formalin, and washings were collected in the original vials with trypsinized cells. Each vial was counted in a Coulter counter to determine the total cell number.

Soft Agar Colony Formation Assay. DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin-streptomycin (complete medium) as detailed above. Soft agar colony formation assay was performed using 6-well plates. Each well contained 2 ml of 0.5% agar in complete medium as the bottom layer, 1 ml of 0.38% agar in complete medium and 1000 cells as the feeder layer, and 1 ml of 0.38% agar in complete medium with either vehicle ethanol or a different dose of silymarin in ethanol as the top layer. Each treatment had three wells. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a group of more than 10 cells were counted as a colony. The rate of colony growth and the optimum time for scoring colonies was assessed by counting colonies at 5, 7, 10, 15, 20, and 30 days and was found to be optimal at day 10. The wells were also examined on day 1 to eliminate possible artifacts caused by any clumps of cells.

RESULTS

Effect of Silymarin on the Activation of erbB1 in DU145 Cells. It has been shown that androgen-independent prostate carcinoma cell lines DU145 and PC3 overexpress erbB1 as well as synthesize and secrete TGF-α, which interacts with erbB1 for autonomous growth through an autocrine feedback loop (16–18). These cell lines, therefore, provide a valuable system to explore the agents that could inhibit erbB1-mediated signaling pathways in PCA. In the present study, we used DU145 cells to assess the inhibitory effects of silymarin on erbB1 activation and its associated downstream events. As shown in Fig. 1*A*, 36 h of serum starvation of DU145 cells resulted in a complete reduction of activated erbB1, as evidenced by no reactivity of immunoprecipitated erbB1 with antiphosphotyrosine antibody in Western blotting (*Lane 1*). On the other hand, treatment of starved cultures with 50 ng/ml TGF-α for 15 min resulted in a highly significant activation of erbB1 as evident by a very strong reactivity with antiphosphotyrosine antibody (Fig. 1*A*, *Lane 2*). Pretreatment of cultures with silymarin during the last 2 h of starvation at the concentrations of 75, 100, and 150 μg/ml followed by treatment with TGF-α at the same dose resulted in a highly significant inhibition of TGF-α-mediated activation of erbB1 in DU145 cells (Fig. 1*A*, *Lanes 4–6*); the concentration of silymarin lower than these did not show such inhibition (Fig. 1*A*, *Lane 3*). To determine whether the decrease in TGF-α-mediated tyrosine phosphorylation of erbB1 by silymarin was due to a decrease in erbB1 expression, the membrane was also probed with anti-EGFR antibody. As shown in Fig. 1*B*, all of the lanes showed equal reactivity of immunoprecipitated erbB1 with anti-EGFR antibody in Western blotting, indicating no change in erbB1 protein levels. Treatment of starved cultures with silymarin alone did not show any effect on tyrosine phosphorylation and protein levels of erbB1 (data not shown).

Effect of Silymarin on the Activation of SHC and the Binding of SHC to erbB1 in DU145 Cells. Activation of erbB1 results in the activation of several different signaling molecules recruited by erbB1 (Refs. 9 and 50–53 and references therein). One such immediate recruitment following erbB1 activation is the adaptor protein SHC (9, 50–53). SHC proteins contain a src homology-2 domain that binds to phosphotyrosine-containing sequences, including those of tyrosine kinase receptors (erbB1 in the present study) upon ligand activation (Refs. 9, 51, 54, and 55 and references therein). We therefore performed the studies to assess whether inhibition of erbB1 activation by silymarin in DU145 cells also impaired the activation of SHC proteins and their binding to erbB1. As shown in Fig. 1*C*, the results obtained for tyrosine phosphorylation of SHC paralleled those for erbB1 acti-

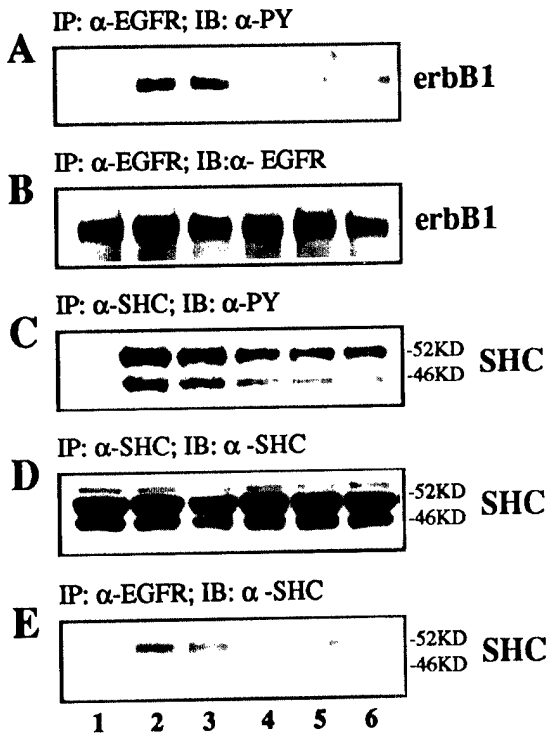

Fig. 1. Effect of silymarin on the activation of erbB1 signaling pathway in DU145 cells. Cells were cultured as described in "Materials and Methods," and at 70–80% confluency, they were serum starved for 36 h. During the last 2 h of starvation, they were treated either with vehicle alone or with varying concentrations of silymarin and, at the end of these treatments, with PBS or TGF-α (50 ng/ml of medium) for 15 min at 37°C. Cell lysates were prepared, erbB1 or SHC was immunoprecipitated using anti-EGFR or anti-SHC antibody, and following SDS-PAGE and Western blotting, membranes were probed with antiphosphotyrosine, anti-EGFR, or anti-SHC antibody and then peroxidase-conjugated appropriate secondary antibody as detailed in "Materials and Methods." Visualization of proteins was done using the ECL detection system. *A*, tyrosine phosphorylation of erbB1; *B*, protein levels of erbB1; *C*, tyrosine phosphorylation of SHC; *D*, protein levels of SHC; *E*, binding of SHC to erbB1. Serum-starved cultures of DU145 cells were as follows: *Lane 1*, vehicle control; *Lane 2*, treated with TGF-α for 15 min; *Lanes 3–6*, treated with 50, 75, 100, and 150 μg/ml of silymarin, respectively, and after 2 h with TGF-α for 15 min. *IP*, immunoprecipitation; *IB*, Western immunoblot.

vation. The starvation of DU145 cells for 36 h resulted in no reactivity of immunoprecipitated SHC to antiphosphotyrosine in Western blot (Fig. 1*C*, *Lane 1*). Treatment of starved cells with TGF-α showed activation of both 52- and 46-kDa SHC proteins, as evidenced by strong reactivity of immunoprecipitated SHC to antiphosphotyrosine (Fig. 1*C*, *Lane 2*). However, treatment of cultures with different doses of silymarin for 2 h prior to the addition of TGF-α showed a highly significant decrease in tyrosine phosphorylation of both 52- and 46-kDa SHC proteins (Fig. 1*C*, *Lanes 3–6*). When the membrane was probed with anti-SHC antibody, there was no change in SHC protein levels (Fig. 1*D*), suggesting that the decrease in SHC activation was not due to a decrease in protein content. To assess the binding of SHC to erbB1, samples were immunoprecipitated with anti-EGFR and immunoblotted with anti-SHC antibody. As shown in Fig. 1*E*, compared to a strong binding in TGF-α alone treated sample (*Lane 2*), silymarin treatment at various doses also resulted in a highly significant decrease in the binding of SHC to erbB1 (*Lanes 3–6*).

Effect of Silymarin on Cell Cycle Regulatory Molecules in DU145 Cells. Defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells (56). Eukaryotic cell cycle progression is regulated by a series of CDKs; their activity is positively regulated by cyclins and negatively regulated by CDKIs (57). The significance of growth factors and the signaling pathway(s) mediated by them to regulate the progression of cell cycle in eukaryotes has been identified as an important component of their function (18, 58, 59). We therefore assessed whether inhibition of erbB1 and SHC activation by silymarin was associated with the alterations in cell cycle regulatory molecules in DU145 cells. As shown in Fig. 2*A*, compared to ethanol-treated controls, treatment of DU145 cells in culture with silymarin resulted in a significant up-regulation of CDKI Cip1/p21 protein levels. The effect of silymarin was both dose- and time-dependent. Maximum up-regulation was observed at 24 h after treatment, because no significant increase was evident after this time (Fig. 2*A*). Similar to Cip1/p21, treatment of DU145 cells with silymarin also resulted in a significant up-regulation in the protein levels of another CDKI, Kip1/p27, which was also dependent on the dose of silymarin as well as the time of treatment (Fig. 2*B*). When the effect of silymarin on the expression of CDKs was assessed, as shown in Fig. 2*D*, silymarin treatment of DU145 cells resulted in a dose- and time-dependent decrease in the protein levels of CDK4. However, no change in the levels of CDK2 (Fig. 2*C*) and CDK6 (Fig. 2*E*) was observed following silymarin treatment up to a concentration of 75 μg/ml for 48 h. Next, we assessed the effect of silymarin on the protein levels of cyclins associated with CDK2 (cyclin E) and with CDK4 and CDK6 (cyclin D1). As shown in Fig. 2, *F* and *G*, treatment of DU145 cells with silymarin did not result in any alterations in the expression of cyclin D1 and cyclin E, respectively, up to 48 h of exposure at a dose of 75 μg/ml.

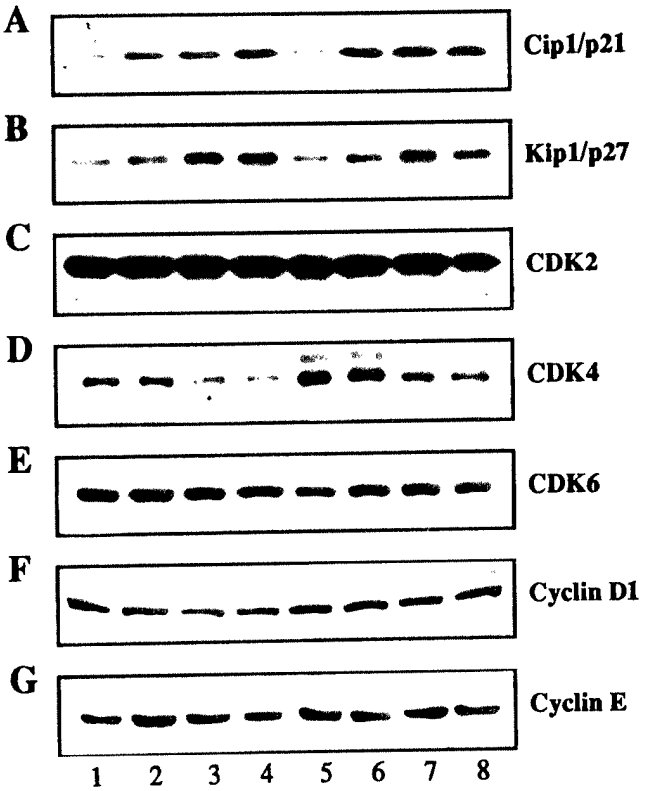

Fig. 2. Effect of silymarin on cell cycle regulatory molecules in DU145 cells. Cells were cultured as described in "Materials and Methods," and at 70–80% confluency (without serum starvation), they were treated with either vehicle alone or varying concentrations of silymarin for 16 h as described in "Materials and Methods." For the studies assessing the time-dependent effect of silymarin, under identical conditions, cultures were treated with 75 μg/ml concentration of silymarin for 8, 24, and 48 h. At the end of these treatments, total cell lysates were prepared and subjected to SDS-PAGE followed by Western blotting as described in "Materials and Methods." Membrane was probed with anti-Cip1/p21 (*A*), Kip1/p27 (*B*), CDK2 (*C*), CDK4 (*D*), CDK6 (*E*), cyclin D1 (*F*), or cyclin E (G) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Cultures of DU145 cells were as follows: *Lanes 1* and *5*, vehicle controls; *Lanes 2–4*, treated with 25, 50, and 75 μg/ml of silymarin, respectively, for 16 h; *Lanes 6–8*, treated with 75 μg/ml of silymarin for 8, 24, and 48 h, respectively.

Because we observed, following silymarin treatment of DU145 cells, a significant increase in the expression of CDKIs Cip1/p21 and Kip1/p27, which bind to and inactivate CDKs (57), we next assessed whether an up-regulation of CDKIs exerted any effect on the kinase activity of CDKs and associated cyclins in silymarin-treated DU145 cells. CDK2 and cyclin E were immunoprecipitated from control and silymarin-treated sample lysates, and kinase activity was determined using histone H1 as substrate. As shown in Fig. 3*A*, compared to vehicle-treated control, silymarin treatment for 16 h resulted in a dose-dependent decrease in CDK2 kinase activity. No change, however, was observed in cyclin E-associated kinase activity (data not shown). For CDK4, CDK6, and cyclin D1-associated kinase activity, these cell cycle regulatory proteins were immunoprecipitated from control and varying doses of silymarin-treated sample lysates, and kinase activity was assessed using a RB-GST fusion protein as a substrate. Similar to CDK2, as shown in Fig. 3*A*, compared to ethanol-treated control, silymarin treatment for 16 h resulted in a significant decrease in CDK4, CDK6, and cyclin D1-associated kinase activity in a dose-dependent manner. To further explore whether the observed decrease in kinase activity of CDKs and cyclin D1 was due to an increase in the binding of CDKs with the induced levels of Cip1/p21 and Kip1/p27 following silymarin treatment, we immunoprecipitated Cip1/p21 and Kip1/p27 from vehicle- and silymarin-treated sample lysates and immunoblotted with anti-CDK2, anti-CDK4, and anti-CDK6 antibodies. As shown in Fig. 3*B*, treatment of DU145 cells with a 75 μg/ml dose of silymarin for 16 h indeed resulted in a significant increase in the binding of all three CDKs (CDK2, CDK4, and CDK6) to Cip1/p21. However, in the case of Kip1/p27, a marked increase in binding was observed only between CDK4 and this CDKI following silymarin treatment (Fig. 3*C*). Together, the data shown in Fig. 3 clearly demonstrate that silymarin treatment of DU145 cells results in a significant decrease in the kinase activity of CDKs and cyclin D1 and that this effect is due to an increased binding of CDKs with the induced levels of CDKIs.

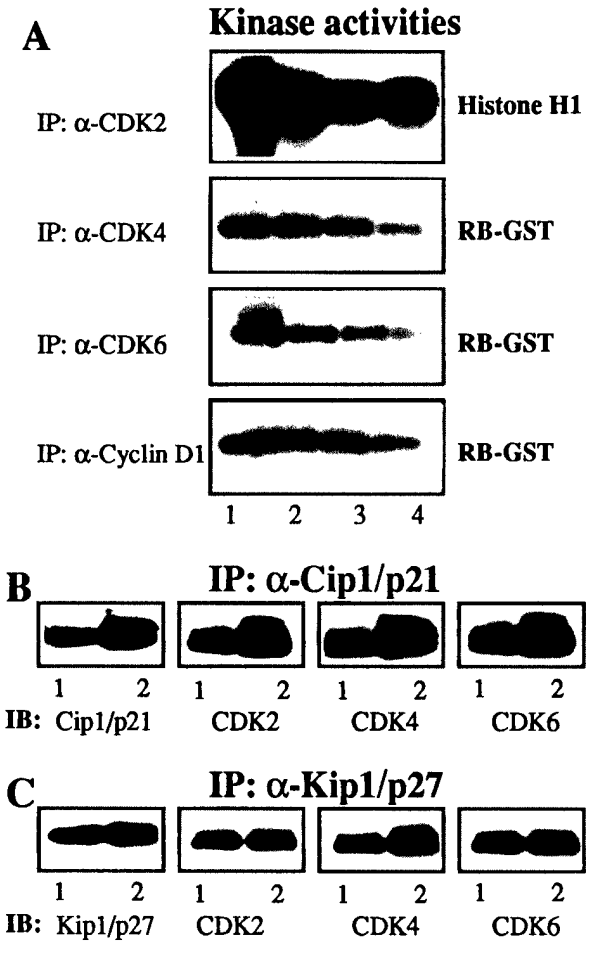

Fig. 3. Effect of silymarin on CDK- and cyclin-associated kinase activities, and binding of CDKIs to CDKs in DU145 cells. Cell culture conditions, treatments, and other details were the same as those described in the legend to Fig. 2. At the end of the treatments, cell lysates were prepared, and CDKs and cyclin-associated kinase activities (*A*), binding of Cip1/p21 to CDKs (*B*), and binding of Kip1/p27 to CDKs (*C*) were determined. CDK2 kinase activity was determined by in-bead histone H1 kinase assay using immunoprecipitated CDK2 from total cell lysates using specific antibody, and CDK4, CDK6, and cyclin D1-associated kinase activity was determined by in-bead RB-GST fusion protein kinase assay using immunoprecipitated CDK4, CDK6, or cyclin D1 from total cell lysates using specific antibody, as described in "Materials and Methods." After the assay, the labeled substrate was subjected to SDS-PAGE, and the gel was dried and exposed to X-ray film. *A*, cultures of DU145 cells were as follows: *Lane 1*, vehicle control; *Lanes 2–4*, treated with 25, 50, and 75 μg/ml of silymarin, respectively, for 16 h. For binding studies, Cip1/p21 or Kip1/p27 was immunoprecipitated and subjected to SDS-PAGE followed by Western blotting, and membrane was probed with anti-Cip1/p21, CDK2, CDK4, or CDK6 antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. *B* and *C*, cultures of DU145 cells were as follows: *Lane 1*, vehicle control; *Lane 2*, treated with 75 μg/ml of silymarin for 16 h. *IP*, immunoprecipitation; *IB*, Western immunoblot.

Effect of Silymarin on Constitutive Activation of erbB1 and Its Association with CDKI Up-Regulation in DU145 Cells. On the basis of the findings that silymarin inhibits TGF-α-mediated activation of erbB1 and its downstream target SHC in serum starved DU145 cells (Fig. 1) and that silymarin also up-regulates the protein levels of CDKIs in DU145 cells grown in 10% serum (Fig. 2), we performed additional studies to identify whether silymarin also inhibits constitutive activation of erbB1 signaling, of which an increase in CDKIs is a plausible downstream result. For these studies, DU145 cells were cultured under normal growth conditions in the presence of 10% serum, and at 70–80% confluence, they were treated either with solvent, different concentrations of silymarin, or mAb 225. The selection of mAb 225 and its dose level in this experiment were based on previous studies showing that treatment of DU145 cells with a 40 nM concentration of mAb 225, a high-affinity ligand blocking anti-EGFR monoclonal antibody, inhibits erbB1 receptor activation and cell growth via an induction of Kip1/p27 and G_1 arrest (16–18). Therefore, mAb 225 is an appropriate and specific erbB1 blocking reagent to be used to compare the effect of and delineate the mechanism associated with silymarin-induced inhibition of erbB1 activation, followed by an increase in the protein levels of CDKIs in DU145 cells. As evidenced by a very strong reactivity with antiphosphotyrosine antibody, erbB1 was found to be highly activated in DU145 cells when they were cultured under normal growth conditions in the presence of 10% serum (Fig. 4*A*, *Lane 1*). However, parallel to the inhibition of TGF-α-caused activation of erbB1 (Fig. 1*A*), treatment of cultures grown in 10% serum with a 50–100 μg/ml dose of silymarin resulted in a significant inhibition of constitutive activation of erbB1 (Fig. 4*A*, *Lanes 3–5*). Whereas observed inhibition at 50 and 75 μg/ml doses of silymarin was comparable to that when mAb 225 was used (Fig. 4*A*, *Lanes 3* and *4 versus Lane 2*), much stronger inhibition of erbB1 activation was evident at a 100 μg/ml dose of silymarin (Fig. 4*A*). As shown in Fig. 4*B*, the observed inhibition in erbB1 activation was not due to a decrease in erbB1 protein levels. Results comparable to those of erbB1 activation were also found for the activation of SHC. As shown in Fig. 4*C*, cells grown in 10% serum showed high levels of constitutive SHC activation, which was inhibited significantly by both mAb 225 and the different doses of silymarin used. There was no change in total SHC protein levels following these treatments (Fig. 4*D*).

When the inhibitory effects of silymarin and mAb 225 on constitutive erbB1 signaling in DU145 cells were analyzed in terms of their association with the up-regulation of CDKI protein levels, striking

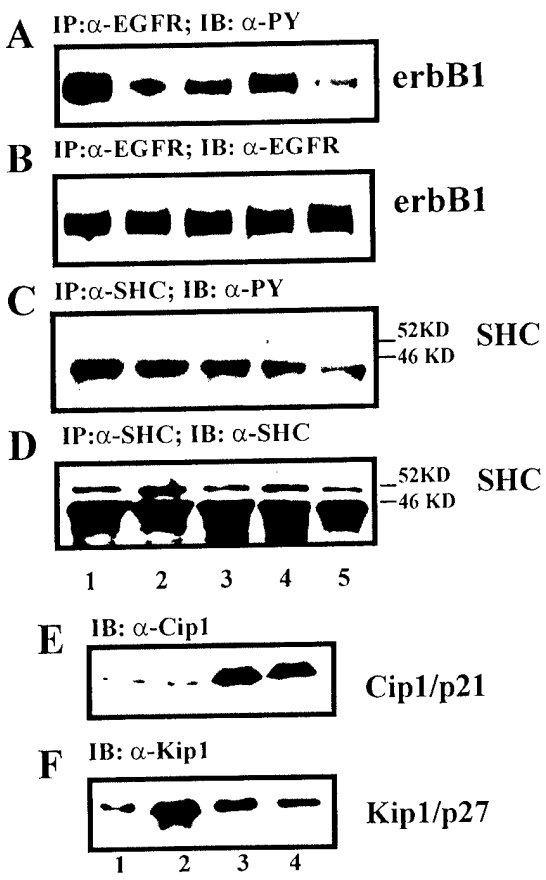

Fig. 4. Effect of silymarin on constitutive activation of erbB1 and its association with CDKI up-regulation in DU145 cells. Cells were cultured as described in "Materials and Methods," and at 70–80% confluency (without serum starvation), they were treated with vehicle alone, mAb 225, or varying concentrations of silymarin for 16 h as described in "Materials and Methods." Cell lysates were prepared, erbB1 or SHC was immunoprecipitated using anti-EGFR or anti-SHC antibody, and following SDS-PAGE and Western blotting, membranes were probed with antiphosphotyrosine, anti-EGFR, or anti-SHC antibody and then peroxidase-conjugated appropriate secondary antibody followed by visualization by the ECL detection system as detailed in "Materials and Methods." *A*, tyrosine phosphorylation of erbB1; *B*, protein levels of erbB1; *C*, tyrosine phosphorylation of SHC; and *D*, protein levels of SHC. In other studies, total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in "Materials and Methods," and the membrane was probed with anti-Cip1/p21 (*E*) or anti-Kip1/p27 (*F*) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Cultures of DU145 cells were as follows: *Lane 1*, vehicle control; *Lane 2*, treated with 6 μg/ml mAb 225; *Lanes 3–5*, treated with 50, 75, and 100 μg/ml of silymarin, respectively, for 16 h. *IP*, immunoprecipitation; *IB*, Western immunoblot.

differences were observed. As shown in Fig. 4, in the same cell lysate samples that were analyzed for erbB1 and SHC activation, only silymarin showed a significant induction in both Cip1/p21 (Fig. 4*E*, *Lanes 3* and *4*) and Kip1/p27 (Fig. 4*F*, *Lanes 3* and *4*) protein levels. However, in the case of mAb 225 treated samples, compared to vehicle-treated control, a significant increase was evident only in the protein level of Kip1/p27 (Fig. 4*F*, *Lane 2*); mAb 225 treatment did not show any change in Cip1/p21 protein level (Fig. 4*E*, *Lane 2*). The observed increase in Kip1/p27 following mAb 225 treatment of DU145 cells is in accord with a recent study showing that mAb 225 treatment induces both mRNA and protein levels of Kip1/p27 and modulates other cell cycle regulatory proteins, leading to a G_1 arrest in cell cycle and inhibition of cell growth in DU145 cells (18). However, to the best of our knowledge, the effect of mAb 225 on the expression of Cip1/p21 in DU145 cells has not been reported earlier, possibly due to a negative finding.

Effect of Silymarin on Cell Cycle Progression of DU145 Cells. Several studies have shown that Kip1/p27 is exclusively associated with G_1 and that CDK4 and CDK6, which exclusively bind to cyclin D, are also associated only with G_1 (Refs. 56, 57, and 60 and references therein). In this situation, the significant up-regulation in the level of Kip1/p27 (in conjunction with Cip1/p21), a decrease in CDK4 expression, and decreased CDK4-, CDK6-, and cyclin D1-associated kinase activity in DU145 cells by silymarin suggested a strong possibility that silymarin treatment of DU145 cells could arrest them in G_1. FACS analysis of ethanol controls and silymarin-treated DU145 cells clearly indicated that a G_1 arrest was induced by silymarin following 24 h of treatment (Fig. 5). This increase in G_1 population by silymarin was accompanied by a large decrease of cells in G_2-M phase of the cell cycle; however, the S population was essentially unchanged (Fig. 5, *middle panel versus left panel*). A further increase in G_1 population was observed at 48 h of silymarin treatment at the same dose that was accompanied by a decrease of cells in both S and G_2-M (Fig. 5, *right panel versus left panel*). Together, these results provide clear evidence that treatment of DU145 cells with silymarin arrests them in G_1 phase of the cell cycle and that this observation was consistent with the results obtained for CDKIs and CDKs in silymarin-treated DU145 cells.

Effect of Silymarin on Anchorage-dependent and Anchorage-independent Growth of DU145 Cells. To assess whether inhibition of erbB1 signaling and alterations in cell cycle regulatory molecules by silymarin are biological effects which occur at similar doses, we next assessed the effect of silymarin on anchorage-dependent and anchorage-independent growth of DU145 cells. In anchorage-dependent cell growth studies, as shown in Fig. 6*A*, the addition of silymarin to exponentially growing DU145 cultures resulted in a significant inhibition of cell growth. The inhibitory effect of silymarin on cell growth was both dose- and time-dependent (Fig. 6*A*). Compared to untreated control (Fig. 6*A*), addition of ethanol vehicle did not result in any alteration in cell growth (data not shown). Treatment of cells at 5–25 μg/ml doses of silymarin showed only marginal inhibition in cell growth during the 6 days of treatment (Fig. 6*A*). A significant inhibition in anchorage-dependent growth of DU145 cells, however, was observed at a dose of 50 μg/ml silymarin during the entire treatment time and accounted for 42% inhibition ($P < 0.001$, Student's t test) after 6 days of treatment (Fig. 6*A*). Although the inhibition at the 50 μg/ml dose of silymarin was even less than 50%, cells stopped growing at this dose as early as after 2 days of treatment because no substantial increase in cell number was evident after this time up to the end of the study (Fig. 6*A*). At much higher doses of silymarin (75 and 100 μg/ml), no cell growth was observed after 1 day of treatment throughout the study (Fig. 6*A*), and in fact, a reduction in

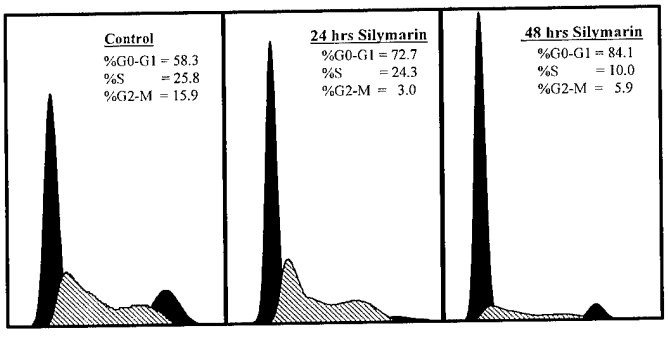

Fig. 5. Effect of silymarin on cell cycle progression of DU145 cells. Cells were cultured as described in "Materials and Methods," and at 70–80% confluency (without serum starvation), they were treated with either vehicle alone or 75 μg/ml concentration of silymarin as described in "Materials and Methods." Twenty-four and 48 h later, cells were trypsinized, and cell pellets were collected as described in "Materials and Methods." The cells were fixed in cold methanol and digested with RNase, and then cellular DNA was stained with propidium iodide as described in "Materials and Methods." Cell cycle distribution was then determined by FACS analysis.

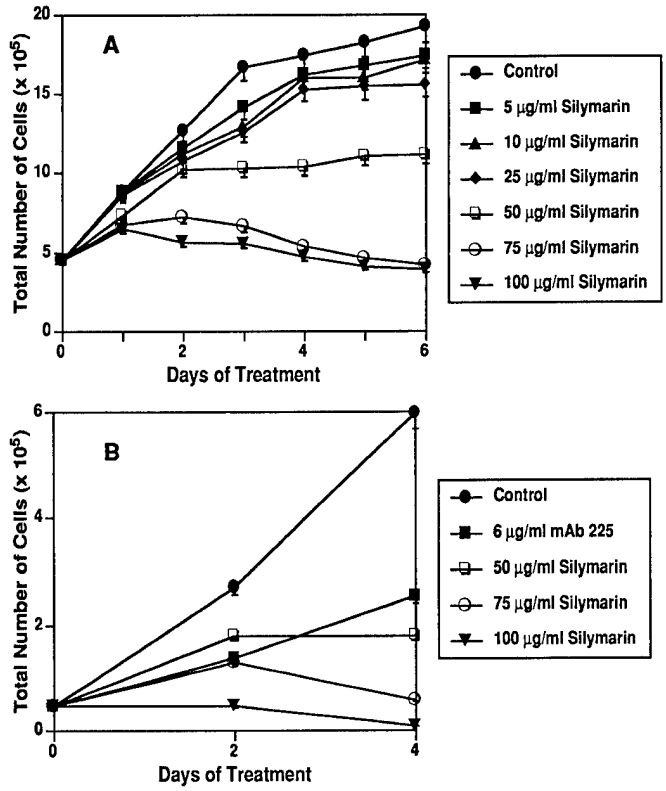

Fig. 6. Effect of silymarin on anchorage-dependent growth of DU145 cells. Cells were cultured as described in "Materials and Methods" and were plated at 0.5×10^5 cells/60-mm plate. For the studies assessing the effect of silymarin on exponentially growing DU145 cell growth (*A*), 2 days after plating, cultures were fed with fresh medium and treated with ethanol vehicle alone or silymarin at the concentrations of 5–100 μg/ml of medium. For the studies comparing the effect of silymarin and mAb 225 on the growth of DU145 cells (*B*), the day after plating, cells were fed with fresh medium and treated with ethanol alone, mAb 225 at a dose of 6 μg/ml of medium, or silymarin at doses of 50, 75, and 100 μg/ml of medium dissolved in ethanol. The cultures were fed with fresh medium with or without same concentrations of mAb 225 or silymarin every other day up to the end of the experiment. At days 1–6 after these treatments, the total number of cells was counted as described in "Materials and Methods." The cell growth data shown are mean ± SE of four independent plates; each sample was counted in duplicate.

initial cell number was evident at these doses of silymarin in a time-dependent manner (Fig. 6*A*).

When the cell growth inhibitory effects of silymarin were compared with those of mAb 225, as shown in Fig. 6*B*, both silymarin and mAb 225 showed highly significant inhibition ($P < 0.001$, Student's t test). However, at 6 μg/ml dose of mAb 225, as much as 50 and 58% inhibition in DU145 cell growth was observed at 2 and 4 days of its treatment, respectively (Fig. 6*B*). These data were consistent with the previous study showing similar growth inhibitory effects of mAb 225 on DU145 cells (18). No further inhibition in cell growth was evident either at twice the dose of mAb 225 or by longer duration of its treatment (data not shown). On the other hand, silymarin treatment at doses of 75 and 100 μg/ml showed 90% and complete inhibition of cell growth at 4 days of treatment (Fig. 6*B*), respectively. Taken together, these results suggest that in the case of silymarin, in addition to inhibition of erbB1 activation, other growth inhibitory pathways are responsible for its strong effect on DU145 cell growth.

On the basis of the results showing dose-dependent inhibition of anchorage-dependent growth of DU145 cells by silymarin, we next assessed the inhibitory effect of silymarin on anchorage-independent growth of DU145 cells by soft agar colony formation assay. First, we determined the optimal time period and number of cells per plate as well as number of cells per colony. In these studies, we found that 1000 DU145 cells grown in soft agar gave rise to optimum colonies of more than 10 cells per colony after 10 days of seeding (data not shown). As many as 40.67 ± 4.2 (mean ± SE of three independent experiments) colonies/1000 cells (per plate) were counted in ethanol-treated controls (data not shown). However, treatment of cells with silymarin resulted in a highly significant inhibition in soft agar colony formation of DU145 cells (Fig. 7). In qualitative analysis, compared to ethanol control (Fig. 7*A*), silymarin treatment at doses of 25 (Fig. 7*B*) and 75 (Fig. 7*C*) μg/ml resulted in a significant decrease in both colony number and size. In quantitative analysis, as shown in Fig. 7*D*, compared to ethanol control, treatment with silymarin at lower doses of 1, 5, 25, and 50 μg/ml resulted in 21% ($P < 0.1$), 38% ($P < 0.01$), 65% ($P < 0.001$), and 80% ($P < 0.001$) inhibition, respectively, in number of colonies per plate. It is important to mention here that these results are significantly different from those observed for anchorage-dependent growth studies, in which low doses of silymarin were not effective in inhibiting cell growth. Results comparable to those of anchorage-dependent growth inhibition by silymarin at higher doses, however, were also observed in soft agar colony formation assay, in which silymarin treatment resulted in 96% ($P < 0.0001$; Fig. 7*D*) and complete inhibition (data not shown) in number of colonies at doses of 75 and 100 μg/ml, respectively.

DISCUSSION

Alarming statistics about human PCA clearly indicate that it is an invasive cancer; behind lung cancer, it is the second leading cause of cancer related deaths among males in the United States (1–3). Geographic variations in the incidence of clinical cancer worldwide are considerable, with an incidence over 100-fold higher in the United States than in China (61, 62). Several studies have suggested that various factors, specifically androgen (testosterone), play a major role in the pathogenesis as well as in the promotion of PCA (2, 4–7). The significance of androgen in prostate carcinogenesis can be attributed by the findings that PCA rarely occurs in eunuchs or men with a deficiency in 5α-reductase, the enzyme responsible for converting testosterone to its active metabolite dihydrotestosterone (Refs. 63–65 and references therein). Because the growth and development of PCA is initially androgen-dependent, androgen deprivation has been extensively explored as a strategy for PCA prevention and therapy (Ref. 65 and references therein). Although PCA patients treated with androgen deprivation therapy often have remission of their PCA, within a few years, tumor regrowth occurs that is largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (Ref. 63 and references therein). When the data are taken together, it can be appreciated that although androgen deprivation is a useful strategy for the prevention and/or therapy of androgen-dependent PCA, additional approaches are needed for advanced and androgen-independent PCA.

In addition to the loss of androgen dependence due to lack of androgen receptor (66), functional autocrine and paracrine growth factor/growth factor receptor interactions are believed to be contributors to the multifactorial mechanisms of androgen independence in PCA cell proliferation (16–18). For example, human prostate carcinoma cell lines PC-3 and DU145, which are derived from androgen-independent tumors and lack androgen receptor (66), express high levels of erbB1 (16–18). In addition, coexpression of erbB1 and TGF-α has been demonstrated in advanced and metastatic PCA (8, 10–14, 67). These studies suggest that a functional autocrine loop may contribute to hormone-independent cancer growth and successful proliferation of PCA at metastatic sites (18) and that agents that could inhibit the activation of RTKs, such as those of erbB family, may be useful for the intervention of PCA. In earlier studies, it has been shown that anti-EGFR monoclonal antibodies 225 and 528 bind to

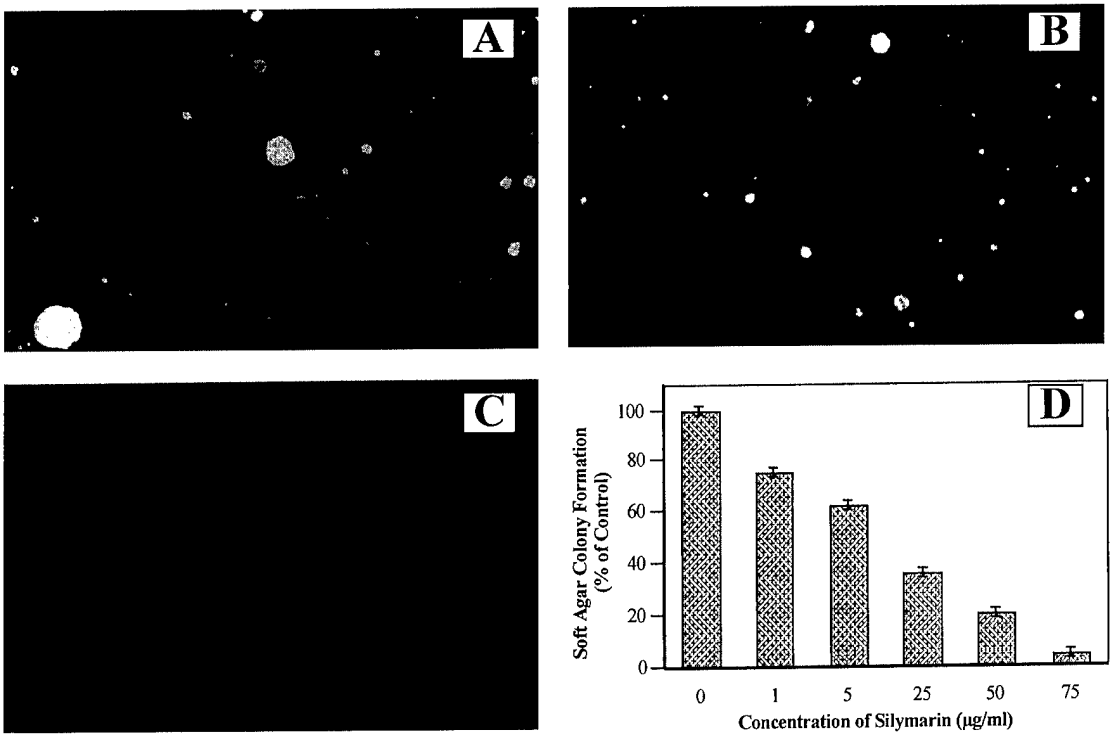

Fig. 7. Effect of silymarin on anchorage-independent growth of DU145 cells. Cells were cultured as described in "Materials and Methods," and a soft agar colony formation assay was performed using 6-well plates as detailed in "Materials and Methods." Each well contained 2 ml of 0.5% agar in complete medium as the bottom layer, 1 ml of 0.38% agar in complete medium and 1000 cells as the feeder layer, and 1 ml of 0.38% agar in complete medium with either vehicle ethanol or different doses of silymarin in ethanol as the top layer. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a group of more than 10 cells was counted as a colony. The data shown are mean ± SE of three independent wells at optimum time of 10 days after the start of cell seeding; the experiment was repeated once with similar results. *A-C*, qualitative analysis of soft agar colony formation assay, where DU145 cells were treated with vehicle alone or with 25 and 75 μg/ml doses of silymarin, respectively. ×100. *D*, quantitative analysis of dose-dependent inhibitory effect of silymarin against soft agar colony formation of DU145 cells.

erbB1 with high affinity, blocking the binding of the ligands EGF and TGF-α to their receptor. This leads to inhibition of erbB1 activation, as well as reduced growth of nontransformed prostatic epithelial cells and human PCA cell lines PC-3 and DU145 (16–18). Comparing the inhibitory effects of (*a*) silymarin on ligand-induced erbB1 activation, and (*b*) both silymarin and mAb 225 on constitutive activation of erbB1 in DU145 cells observed in the present study to those reported with anti-EGFR antibody (18), there is a possibility that silymarin also directly affects the erbB1, leading to an inhibition in the binding of its ligand TGF-α. More detailed studies, however, are needed to address this possibility.

Stimulation of erbB1 kinase activity is known to result in the activation of several different signaling molecules, as well as the recruitment of adaptor proteins (9, 50–53). One such recruitment following erbB1 activation is SHC, which, following tyrosine phosphorylation, acts as an adaptor for other src homology-2-containing proteins in the signal transduction pathway(s) (9, 50–55). Being one of the immediate downstream signaling molecules recruited by erbB1, inhibition of ligand-induced, as well as endogenous, activation of erbB1 by silymarin also resulted in a significant decrease in SHC tyrosine phosphorylation, concomitant with a decrease in its binding to erbB1, suggesting that SHC is also targeted by silymarin via erbB1. To the best of our knowledge, ours is the first study showing the involvement of SHC in erbB1 signaling and its inhibitory modulation by an anticarcinogenic agent in human prostate carcinoma cell line DU145. More detailed studies, however, are needed with other agents to explore the modulation of erbB1-SHC downstream events in human PCA.

The significance of growth factors and the signaling pathway(s) initiated by them to regulate cell cycle progression in eukaryotes has been identified as an important component of their function (Refs. 56–60 and 68 and references therein). Several studies have shown that cell signaling pathways determine cell growth and inhibition through cell cycle regulation (56–60, 68). However, cancer cells often display abnormalities in genes that govern the responses of these cells to external growth factors; growth factors receptors; proteins involved in the pathways of signal transduction in the cytoplasm, the nucleus, or both; and nuclear transcription factors (68). In addition, defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells (56). Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases (69–71). The activities of CDKs are regulated positively by cyclins and negatively by CDKIs (57). It is becoming increasingly clear that cyclin D1 and its associated CDK4 normally control cell cycle events in G_1, and cyclin E associated with CDK2 mediates late G_1 to early S (71–74). The *cyclin D1* gene is turned on at G_1 phase upon receipt of a cell cycle activation signal by growth factors (56, 60, 68). The cyclin D1 protein then binds with CDK4 (it is also known to bind with CDK2 and CDK6), and this complex hyperphosphorylates RB leading to its release from E2F (56, 59, 60, 68). The free transcription factor E2F then activates c-myc, resulting in cellular proliferation by progression through G_1 (56, 59, 60, 68). The CDK activity, however, is negatively regulated by CDKIs (57, 75). In general, the relative abundance of CDKIs present at any point in the cell cycle sets thresholds for CDK-cyclin activation that must be overcome for the cell cycle to proceed (56, 57, 60, 68, 75). Impairment of a growth-stimulatory signaling pathway (such as erbB1, raf, or mitogen-activated protein kinase) has been shown to modulate the expression of CDKIs such as Cip1/p21 and Kip1/p27 (18, 49, 58, 59). An induced

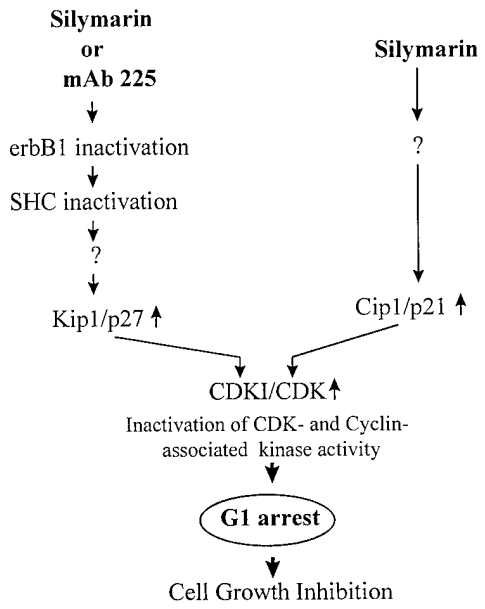

Fig. 8. Proposed mechanisms of the dual growth inhibitory pathways mediated by silymarin in DU145 cells involving erbB1-SHC inactivation followed by Kip1/p27 induction, and an unknown target of silymarin leading to Cip1/p21 up-regulation.

CDKI binds to and subsequently inhibits cyclin-CDK activity, which interferes with hyperphosphorylation of RB, keeping it in the hypophosphorylated form and bound to E2F, thereby blocking cell proliferation and inducing cell growth arrest (56, 60, 68). A decrease in the levels of cyclins and/or associated CDKs also leads to an arrest in cell cycle progression at a specific phase(s), depending on the particular regulatory molecule involved (56, 57, 60, 68).

In the present study, we observed that silymarin, which inhibited both ligand-induced and constitutive activation of erbB1 and its downstream signaling events, also up-regulated the levels of CDKIs Cip1/p21 and Kip1/p27 and that the increased CDKI levels were largely responsible for a marked decrease in the kinase activity of CDKs due to their increased binding with CDKIs. In addition, silymarin treatment of DU145 cells also resulted in a significant decrease in the levels of CDK4. Consistent with the findings of alterations in cell cycle regulatory proteins, silymarin induced an arrest in G_1 and inhibited both anchorage-dependent and anchorage-independent growth of human prostate carcinoma cell line DU145. However, when the results obtained with silymarin were compared with those when cells were treated with mAb 225, striking differences were observed in terms of CDKI up-regulation and inhibition of cell growth. Treatment of DU145 cells with mAb 225 resulted in the induction of Kip1/p27 only, not Cip1/p21. In addition, mAb 225 treatment resulted in almost 50% inhibition in cell growth, but silymarin showed complete inhibition. Whereas it would have been argued that the inhibitory effect of silymarin on cell growth is dose-dependent and higher doses show complete inhibition, doses of mAb 225 even two times higher did not result in any further inhibition of cell growth. Furthermore, the data obtained with mAb 225 in the present study were in accord with an earlier report showing that mAb 225 treatment results in G_1 arrest and up-regulation of CDKI Kip1/p27 together with modulation of other cell cycle regulatory molecules and causes about 50% inhibition of cell growth in human prostate carcinoma cell line DU145 (18).

When the data are taken together, it can be argued that erbB1 receptor inactivation by blocking antibody leads to partial inhibition of cell growth as a biological response and that other mechanisms are still operational and responsible for DU145 cell growth independent of erbB1 signaling pathway. The results obtained in the present study, in conjunction with those reported earlier (18), clearly show that specific blocking of erbB1 receptor activation leads to an up-regulation of Kip1/p27 but not Cip1/p21, suggesting that silymarin possesses at least two independent growth inhibitory pathways. As shown by a schematic representation in Fig. 8, we suggest that, like mAb 225, silymarin inactivates erbB1-SHC signaling pathway leading to up-regulation of Kip1/p27 followed by its increased binding with CDK causing a decrease in CDK- and cyclin-associated kinase activity. This leads to a G_1 arrest and resultant cell growth inhibition. In addition, by a mechanism not yet known at this point, silymarin also induces the expression of Cip1/p21 via an erbB1 inactivation-independent pathway that is presumably responsible for the remainder of the cell growth inhibitory potential of silymarin in DU145 cells. Further studies are needed to identify the targets associated with the up-regulation of Cip1/p21 by silymarin and to define whether the effect of silymarin observed in DU145 cells is a specific phenomenon or also occurs in other malignant cells. However, in summary, based on the findings reported here, it can be concluded that silymarin may exert a strong anticarcinogenic effect against human PCA.

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Appendix 2

Silibinin decreases prostate-specific antigen with cell growth inhibition via G_1 arrest, leading to differentiation of prostate carcinoma cells: Implications for prostate cancer intervention

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ABSTRACT **Reduction in serum prostate-specific antigen (PSA) levels has been proposed as an endpoint biomarker for hormone-refractory human prostate cancer intervention. We examined whether a flavonoid antioxidant silibinin (an active constituent of milk thistle) decreases PSA levels in hormone-refractory human prostate carcinoma LNCaP cells and whether this effect has biological relevance. Silibinin treatment of cells grown in serum resulted in a significant decrease in both intracellular and secreted forms of PSA concomitant with a highly significant to complete inhibition of cell growth via a G_1 arrest in cell cycle progression. Treatment of cells grown in charcoal-stripped serum and 5α-dihydrotestosterone showed that the observed effects of silibinin are those involving androgen-stimulated PSA expression and cell growth. Silibinin-induced G_1 arrest was associated with a marked decrease in the kinase activity of cyclin-dependent kinases (CDKs) and associated cyclins because of a highly significant decrease in cyclin D1, CDK4, and CDK6 levels and an induction of Cip1/p21 and Kip1/p27 followed by their increased binding with CDK2. Silibinin treatment of cells did not result in apoptosis and changes in p53 and bcl2, suggesting that the observed increase in Cip1/p21 is a p53-independent effect that does not lead to an apoptotic cell death pathway. Conversely, silibinin treatment resulted in a significant neuroendocrine differentiation of LNCaP cells as an alternative pathway after Cip1/p21 induction and G_1 arrest. Together, these results suggest that silibinin could be a useful agent for the intervention of hormone-refractory human prostate cancer.**

Prostate cancer (PCA) is the most common invasive malignancy and second leading cause of cancer deaths in United States males (1). Clinical PCA incidence is low in Asians and highest in African-Americans and Scandinavians (2, 3). However, once moved to the United States, incidence and mortality because of PCA increase in Asians, approximating those of Americans (3). Epidemiological studies suggest that dietary and environmental factors are major causes for an increase in PCA (2, 3). Low-fat and high-fiber diets significantly affect sex hormone metabolism in men (4). In Japan and other Asian countries, despite the same incidence of latent small or noninfiltrating PCA, mortality rate is low (3). This could be explained, at least partly, by a diet-related lowering of biologically active androgen (4). The importance of androgen in PCA also is suggested by the observations that PCA rarely occurs in eunuchs or men with deficiency in 5α-reductase, the enzyme that converts testosterone to its active metabolite 5α-dihydrotestosterone (DHT) (5). In addition, at least 75% of PCAs with metastatic potential are androgen-dependent at initial diagnosis (6).

Androgen receptors (ARs) are required for development of both normal prostate and PCA (7). A high proportion of mutations are shown in the ligand-binding domain of AR in hormone-refractory and metastatic PCA (7), and mutant ARs could be activated by estrogen and progesterone (7). Changes in specificity of AR may provide a selective advantage in metastatic androgen-independent PCA because they remain active after androgen ablation (7). A notable gene regulated by androgen in normal prostate and PCA cells is prostate-specific antigen (PSA) (8). PSA is demonstrated to be a sensitive and specific tumor marker for PCA screening and assessment (9) and is used as an indicator of disease and response to PCA therapy (10). Several trials also have shown a direct relationship between decline in PSA and shrinkage of PCA (11). Whereas stimulation of mutant AR in human PCA LNCaP cells by androgen does not differ from stimulation of wild-type AR, estrogenic substance and some antiandrogens bind to AR in LNCaP cells with higher affinity, efficiently stimulate its transactivation function, and increase PSA (7).

Traditional Asian diets are low in animal proteins and fat, high in starch and fiber, and rich in "weak plant estrogens," which are released in large amounts in urine and serum (12, 13). Some of these phytoestrogens possess weak estrogenic, antiestrogenic, and antioxidant activity, and, therefore, possess the potential for exerting an influence on hormone-dependent cancers including PCA (12, 13). Two groups of phytoestrogens, polyphenolic flavonoid antioxidants and lignans, are receiving attention for the prevention and intervention of human cancers including PCA (12–14). Silymarin, a polyphenolic flavonoid isolated from the seeds of milk thistle (*Silybum marianum*), is composed mainly of silibinin (or silybin; Fig. 1*A*), with small amounts of other stereoisomers isosilybin, dihydrosilybin, silydianin, and silychristin (15). Silymarin and silibinin have human acceptance, being used clinically in Europe and Asia for the treatment of liver diseases (reviewed in refs. 16–19). Human populations in Europe have been using silymarin or silibinin in a whole range of liver conditions (16, 17). As therapeutic agents, both silymarin and silibinin are well tolerated and largely free of adverse effects (15–19). Silymarin is sold in the United States and Europe as a dietary supplement, and silibinin is used clinically as silipide, a lipophilic silibinin–phosphatidylcholine complex (16).

Recently, we showed that silymarin affords high to complete protection against tumorigenesis in mouse skin models (18, 19). Likewise, in a mammary gland culture initiation–promotion protocol, silymarin inhibits tumor promotion (19). More recent studies by us found that both silibinin and silymarin possess comparable inhibitory effects on human carcinoma cell growth

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Abbreviations: AR, androgen receptor; CDK, cyclin-dependent kinase; CDKIs, CDK inhibitors; DHT, 5α-dihydrotestosterone; EC, electrochemical; cFBS, charcoal-stripped FBS; K8 & K18, cytokeratins 8 and 18; PCA, prostate cancer; PSA, prostate-specific antigen; RB, retinoblastoma.

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and DNA synthesis and are equally strong antioxidants (R.A. and colleagues, unpublished observations). Based on (*i*) structural similarity of silibinin with phytoestrogens for a polyphenolic flavonoid skeleton, (*ii*) strong antioxidant and anticarcinogenic effects of silibinin, (*iii*) the fact that silibinin is used clinically and marketed as dietary supplement, and (*iv*) the bioavailability of silibinin in prostate after its oral administration to mice (R.A. and colleagues, unpublished observations), we reasoned that silibinin also could be a useful agent for the intervention of human PCA. Here, we show that silibinin decreases intracellular and secreted levels of PSA in human PCA LNCaP cells under both serum- and androgen-stimulated conditions concomitant with inhibition of cell growth via a G_1 arrest in cell cycle progression. The G_1 arrest by silibinin does not lead to apoptosis but causes neuroendocrine differentiation of the cells.

MATERIALS AND METHODS

Cells and Cultures. Human prostate carcinoma LNCaP cells and NIH 3T3 cells were obtained from American Type Culture Collection. Normal human epithelial prostate cells were from Clonetics (San Diego). LNCaP and NIH 3T3 cells were cultured in RPMI 1640 medium and DMEM, respectively, with 10% FBS and 1% penicillin-streptomycin (P-S). LNCaP cells also were cultured in 10% charcoal-stripped FBS (cFBS) and 1% P-S with or without 1 nM DHT. Normal prostate cells were cultured in defined medium as suggested by the vendor.

Silibinin and Its Purity. Silibinin (Fig. 1*A*), International Union of Pure and Applied Chemistry name: 3,5,7-trihydroxy-2-[3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-1,4-benzodioxan-6-yl]-4-chromanon, was from U. Mengs (MADAUS AG, Cologne, Germany) and Sigma. Purity of silibinin from both sources was checked by HPLC equipped with UV followed by electrochemical detectors (EC). The HPLC system consisted of two ESA 580 pumps, an ESA RP-C18 column (3 mm, 4.6 × 250 mm), a UV detector (at λ 270 nm), an EC detector (at 500 mV potential), and an ESA 5600 control and analysis software. HPLC mobile phase contained solvent A [7.5% methanol in 100 mM of acetate buffer with 50 mM of triethylamine (TEA)/1 mM of 1-octanesulfonic acid (OSA), pH 4.8] and solvent B (80% methanol in 100 mM of acetate buffer with 50 mM TEA/1 mM OSA, pH 4.8). The linear gradient, at 0.6 ml/min, was 0–5 min, 75% A and 25% B; 5–15 min, 50% of both A and B; 15–20 min, 30% A and 70% B; 20–25 min, isocratic 30% A and 70% B; and 25 min, stop of run. Column eluate was monitored at 270 nm followed by EC detection. As shown in Fig. 1*B*, using these HPLC conditions, silibinin showed a single peak in both 270 nm UV and EC detections, with a retention time of 13.5 min. These HPLC profiles also show the purity of silibinin to be 100%.

Silibinin Treatments. Silibinin was dissolved in ethanol. Final volume of ethanol in culture during silibinin treatment and controls did not exceed 0.5%. LNCaP cells were grown in 10% FBS to 80% confluency and treated with ethanol or varying doses

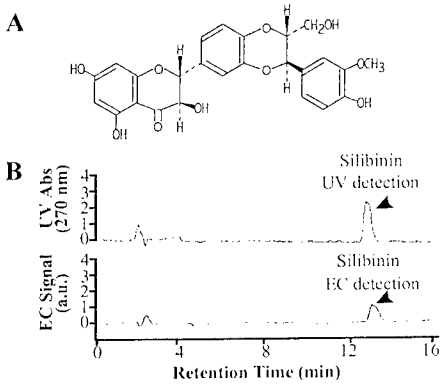

FIG. 1. Chemical structure of silibinin (*A*) and HPLC profiles of silibinin by UV and EC detection (*B*).

of silibinin for 20 hr or 75 μg/ml of silibinin for varying times. Cells also were treated with paclitaxel (1 μM final concentration) for 20 hr. Cells then were lysed in 0.5 ml lysis buffer as detailed recently (20). In another study, cells grown in 10% FBS were treated with ethanol or 25 and 75 μg/ml of silibinin for 24, 48, and 72 hr, and medium was collected. Cells also were grown in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cell lysates then were prepared (20).

Western Blotting and Kinase Assays. Levels of PSA, cell cycle and apoptosis regulatory molecules, cytokeratins 8 and 18 (K8 & K18), and chromogranin A were determined by Western blotting. Equal amounts of protein (10–80 μg) from cell lysate or 20 μl of medium sample was denatured in sample buffer and subjected to SDS/PAGE on a 12% gel, and proteins were transferred onto membrane. The blots were probed with specific primary followed by secondary antibody and visualized by enhanced chemiluminescence. The binding of cyclin-dependent kinase inhibitors (CDKIs) with CDKs, CDK2- and cyclin E-H1 histone kinase activity, and CDK4-, CDK6-, and cyclin D1-retinoblastoma (RB) kinase activity were determined as detailed recently (20).

Cell Growth Assay. LNCaP cells were plated at 1×10^4 cells per 60-mm plate in RPMI 1640 medium containing 10% FBS. To assess the effect of silibinin on normal cell growth, NIH 3T3 cells were plated at the same density, and normal human prostate cells were plated at 2,500 cells/cm^2. On day 2, cells were fed with fresh medium and treated with ethanol or varying doses of silibinin (5, 25, 50, and 75 μg/ml). The cultures were fed with fresh medium with the same treatments on alternate days. After 1–6 days of treatments, cells were trypsinized and counted (20). In other studies, LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cells then were collected and counted (20). To assess cytotoxicity of silibinin, cell viability was determined by Trypan blue assay.

FACS Analysis. LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 24 hr, were treated with ethanol or 50 μg/ml of silibinin. Cells then were trypsinized, and cell cycle distribution was analyzed as detailed recently (20).

DNA Ladder Assay. LNCaP cells at 70–80% confluency were treated with different doses of silibinin for 24 and 48 hr, and, thereafter, trypsinized cells (together with any floating cells) were collected. The DNA ladder analysis then was done as detailed recently (21).

Morphological Analysis. LNCaP cells were cultured in 10% FBS or 10% cFBS without or with 1 nM DHT for 5 days and, during the last 48 hr, were treated with ethanol or 50 μg/ml of silibinin. Pictures then were taken by using a phase-contrast microscope at ×200 magnification.

RESULTS

Silibinin Decreases Serum- and DHT-Stimulated PSA Expression in LNCaP Cells. PSA has its acceptance and approval from FDA as a screening tool for human PCA. Therefore, to evaluate the usefulness of silibinin for PCA intervention, we assessed its effect on PSA levels in LNCaP cells. Consistent with an earlier study (8), LNCaP cells showed high levels of intracellular PSA as evidenced by a 33- to 34-kDa band (Fig. 2*A*). However, treatment of cells grown in 10% FBS with silibinin resulted in a highly significant decrease in intracellular PSA levels in a dose- and time-dependent manner (Fig. 2*A*). In a quantitative analysis, 50, 75, and 100 μg/ml of silibinin showed 54, 66, and 79% reduction in intracellular PSA levels, respectively. Similarly, cells grown in 10% FBS with 25 and 75 μg/ml of silibinin for 24 and 48 hr also showed a significant decrease in secreted PSA (Fig. 2*B*). Silibinin treatment for 24 hr at 25- and 75-μg/ml doses led to a 45 and 59% reduction in PSA secretion in medium, respectively. Because promoter of PSA gene contains functional androgen-responsive element (8) and DHT increases PSA production in LNCaP cells

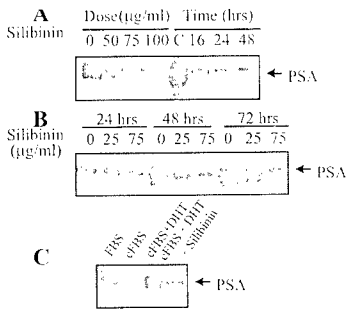

FIG. 2. Silibinin decreases serum- and DHT-stimulated PSA expression in LNCaP cells. (*A*) Effect of silibinin on intracellular PSA in cells grown in 10% FBS. Cells were treated with silibinin for 20 hr or for indicated times at 75 μg/ml; C, control cells treated with ethanol for 48 hr. (*B*) Effect of silibinin on secreted (medium) PSA in cells grown in 10% FBS. Cells were treated with silibinin for the indicated doses and time, and medium was collected. (*C*) Effect of silibinin on intracellular PSA in cells grown in 10% cFBS + 1 nM DHT. Cells were grown in: 1, 10% serum; 2, 10% cFBS; 3, 10% cFBS + 1 nM DHT; or 4, 10% cFBS supplemented with 1 nM DHT + 50 μg/ml of silibinin, and cell lysates were prepared. The data in *C* are at 5 days of cultures; silibinin was added at day 4. PSA protein levels were determined in cell lysates and medium as detailed in *Materials and Methods*. The Western blot data shown are representative of three independent experiments with similar findings.

(7), we next examined whether inhibitory effects of silibinin on PSA levels are mediated via AR. Compared with cells grown in 10% FBS showing strong PSA levels, cells grown in 10% cFBS showed no reactivity for PSA protein (Fig. 2*C*). However, cells grown in 10% cFBS + 1 nM DHT showed levels of PSA comparable to that for 10% FBS (Fig. 2*C*). Treatment of cells grown in 10% cFBS and 1 nM DHT with 50 μg/ml of silibinin resulted in a 56% reduction in DHT-stimulated intracellular PSA levels (Fig. 2*C*).

Silibinin Inhibits Serum- and DHT-Stimulated Growth of LNCaP Cells with No Effects on Normal Cells. To assess whether an observed decrease in PSA by silibinin is a biological response, we examined its effect on LNCaP cell growth. Treatment of cells grown in 10% FBS with silibinin resulted in a highly significant to complete inhibition of their growth in both a dose- and time-dependent manner (Fig. 3*A*). An inhibitory effect of silibinin was evident at 2 days, but a more profound effect was observed during 4–6 days of treatment. The 5- and 25-μg/ml doses of silibinin showed 42 and 61% inhibition in cell growth, respectively (Fig. 3*A*). Cells treated with 50 and 75 μg/ml of silibinin showed 93% and complete growth inhibition, respectively (Fig. 3*A*). At these doses of silibinin, cells stopped growing as early as 1 and 2 days, with a small reduction in initial cell number at 75 μg/ml (Fig. 3*A*). In studies assessing the effect of silibinin on androgen-stimulated growth of LNCaP cells, compared with cells grown in 10% FBS, cells grown in 10% cFBS showed a 68% reduction in growth (Fig. 3*B*). This was an expected finding because cFBS is devoid of hormones and other growth agents. Cells grown in 10% cFBS + 1 nM DHT showed much higher growth, but it was only 77% of that observed in 10% FBS (Fig. 3*B*). Silibinin treatment, however, showed 38% inhibition of DHT-stimulated cell growth (Fig. 3*B*). Together, the inhibitory effects of silibinin on FBS- and DHT-stimulated LNCaP cell growth were consistent with a decrease in PSA levels. Silibinin, however, did not show a considerable inhibition of NIH 3T3 and normal human prostate cell growth (data not shown). In cell viability, silibinin did not show cytotoxicity at present doses (data not shown).

Silibinin Induces G_1 Arrest and Decreases CDK and Cyclin Kinase Activity in LNCaP Cells. We next assessed whether cell growth-inhibitory effects of silibinin are via perturbation in cell cycle progression. Fluorescence-activated cell sorter (FACS) analysis of control and silibinin-treated cells grown in 10% FBS clearly indicated a G_1 arrest by silibinin (Fig. 4). The increase in

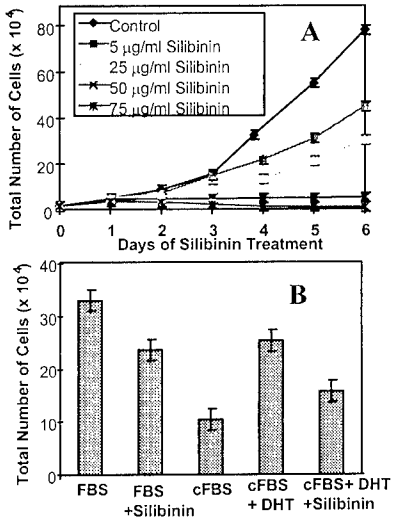

FIG. 3. Silibinin inhibits serum- and DHT-stimulated growth of LNCaP cells. (*A*) Dose- and time-dependent inhibitory effect of silibinin on serum-stimulated cell growth. Cells were treated with ethanol (control) or indicated doses of silibinin. (*B*) Inhibitory effect of silibinin on DHT-stimulated cell growth. Cells were grown in FBS, 10% serum; FBS + silibinin, 10% serum + 50 μg/ml of silibinin; cFBS, 10% cFBS; cFBS + DHT, 10% cFBS + 1 nM DHT; or cFBS + DHT + silibinin, 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin. The data in *B* are at 5 days of cultures; silibinin was added at day 4. After desired treatments, cells were trypsinized and counted as described in *Materials and Methods*. Each data point represents mean ± SE of four independent plates; each sample was counted in duplicate.

G_1 population by silibinin (82.8 vs. 63% in control) was accompanied by a large decrease of cells in both S and G_2/M phases (Fig. 4 *B* vs. *A*). G_1 arrest by silibinin also was found at other time points (data not shown). Similar to silibinin, when cells were

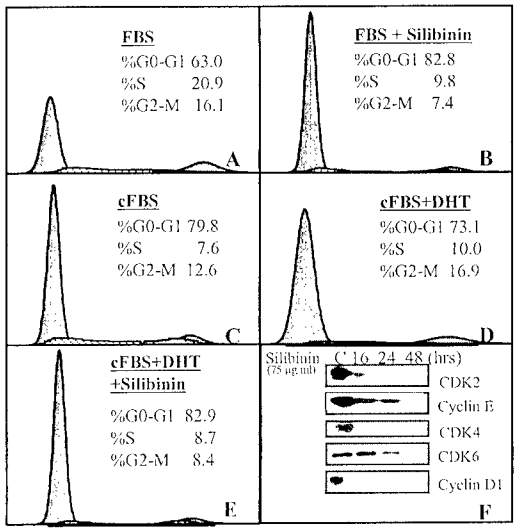

FIG. 4. Silibinin induces G_1 arrest and decreases CDK and cyclin-associated kinase activity in LNCaP cells. Cell cycle phase distribution of LNCaP cells grown in 10% serum (*A*); 10% serum + 50 μg/ml of silibinin (*B*); 10% cFBS (*C*); 10% cFBS + 1 nM DHT (*D*); and 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin (*E*). The data are at 5 days of cultures; silibinin was added at day 4. After desired treatments, cells were trypsinized and FACS analysis was done as described in *Materials and Methods*. (*F*) Inhibitory effect of silibinin on CDK and cyclin kinase activity. Cells were treated with 75 μg/ml of silibinin for the indicated time, and CDK and cyclin kinase activity was determined as described in *Materials and Methods*; C, control cells treated with ethanol for 48 hr. The cell cycle phase distribution and kinase activity data shown are representative of three independent experiments with similar findings.

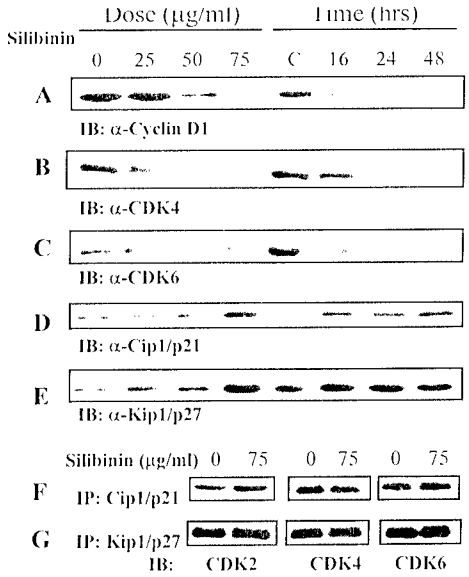

FIG. 5. Silibinin modulates protein levels of cyclin D1, CDKs, and CDKIs and increases binding of CDKIs to CDK2 in LNCaP cells. Dose- and time-dependent effect of silibinin on levels of cyclin D1 (*A*); CDK4 (*B*); CDK6 (*C*); Cip1/p21 (*D*); and Kip1/p27 (*E*). Cells were treated with silibinin for 20 hr or for the indicated time at 75 μg/ml; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared and subjected to SDS/PAGE, Western blotting, and enhanced chemiluminescence detection as described in *Materials and Methods*. Shown also is the effect of silibinin on binding of CDKs with Cip1/p21 (*F*) and Kip1/p27 (*G*). Cells were treated with vehicle or 75 μg/ml of silibinin for 16 hr, and cell lysates were prepared. CDKIs binding with CDKs was determined as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

grown in 10% cFBS, a G_1 arrest also was observed (Fig. 4 *C* vs. *A*). This finding suggests a possibility that observed G_1 arrest by silibinin may be due to its inhibitory effect on growth-stimulating factors that are not present in cFBS. Additional studies also were performed to answer two questions: first, whether absence of androgen in cFBS was a major factor for observed G_1 arrest in 10% cFBS grown cells and, second, whether silibinin inhibits DHT-stimulated cell cycle progression. Compared with 10% cFBS, cells grown in 10% cFBS + 1 nM DHT showed a release from G_1 arrest (Fig. 4 *D* vs. *C*). However, when FACS data for 10% cFBS + 1 nM DHT were compared with 10% FBS, DHT-stimulated release from G_1 arrest in 10% cFBS cells was not complete (Fig. 4 *D* vs. *A*). DHT-stimulated release of cells from G_1 arrest, however, was blocked completely by silibinin (Fig. 4 *D* vs. *E*). Together, these data suggest that, in addition to androgen, there are other growth factors in serum responsible for growth and cell cycle progression of LNCaP cells and that silibinin results in a G_1 arrest in cell cycle progression of cells that are stimulated for growth by serum or only androgen.

Cell cycle progression is regulated via irreversible transitions propelled by CDKs and cyclins (22, 23). Whereas CDK4 (or CDK6)/cyclin D1 are involved in early G_1 phase, transition from G_1 to S is regulated by CDK2/cyclin E (23). Therefore, we reasoned that observed G_1 arrest by silibinin could be due to a decrease in kinase activity of CDKs and cyclins. Indeed, 75 μg/ml of silibinin showed a time-dependent decrease in CDK2 and cyclin E kinase activity (Fig. 4*F*); at 48 hr, kinase activity was not detectable in both cases. Similarly, silibinin also resulted in a highly significant decrease in CDK4, CDK6, and cyclin D1 kinase activity (Fig. 4*F*). Together, these data suggest that G_1 arrest induced by silibinin is due to a significant decrease in kinase activity of both CDKs and cyclins associated with early G_1 phase and late G_1- to S-phase transition.

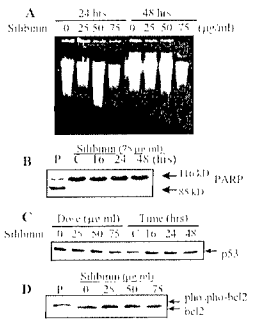

FIG. 6. Silibinin does not induce apoptosis and modulation of p53 and Bcl2 in LNCaP cells. (*A*) Agarose gel electrophoresis of cellular DNA showing a lack of DNA ladder by silibinin treatment. Cells, at 80% confluency, were treated with silibinin for the indicated doses and time. Cells were collected and cellular DNA was isolated, followed by agarose gel electrophoresis as described in *Materials and Methods*. (*B*) A lack of silibinin's effect on PARP cleavage. Cells were treated with paclitaxel (P) for 20 hr at 1 μM or for the indicated time at 75 μg/ml of silibinin; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and PARP protein level and cleavage were detected as described in *Materials and Methods*. (*C*) Dose- and time-dependent effect of silibinin on p53 expression. Cells were treated with silibinin for 20 hr or for the indicated time at 75 μg/ml of silibinin; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and p53 levels were detected as described in *Materials and Methods*. (*D*) Dose-dependent effect of silibinin on bcl2 expression. Cells were treated with paclitaxel (P) for 20 hr at 1 μM or silibinin for 20 hr, cell lysates were prepared, and bcl2 levels were detected as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

Silibinin-Induced Decrease in Kinase Activity of CDKs and Cyclins Is Mediated via a Decrease in Cyclin D1, CDK4, and CDK6 Levels and an Induction of Cip1/p21 and Kip1/p27 and Their Increased Binding with CDK2 in LNCaP Cells. CDK activity is regulated positively by cyclins and negatively by CDKIs (22, 23). Based on silibinin's effect on kinase activity, we assessed its effect on (*i*) CDK and cyclin levels and (*ii*) CDKI Cip1/p21 and Kip1/p27 levels and their binding with CDKs. Silibinin resulted in a significant to complete reduction in cyclin D1 protein (Fig. 5*A*) and showed a strong decrease in CDK4 and CDK6 (Fig. 5 *B* and *C*). No effect of silibinin, however, was evident on CDK2 and cyclin E (data not shown). In other studies, silibinin resulted in both dose- and time-dependent induction of CDKIs Cip1/p21 (Fig. 5*D*) and dose-dependent induction of Kip1/p27 (Fig. 5*E*); maximum increase was evident at 24 and 16 hr, respectively. Because an induction in CDKI normally leads to an increase in its binding to and subsequent inactivation of CDK-cyclin complex (22, 23), we also investigated whether an observed decrease in CDK and cyclin kinase activity also is due to an increased CDK binding with up-regulated Cip1/p21 and Kip1/p27 by silibinin. As shown in Fig. 5 *F* and *G*, silibinin resulted in an increase only in CDK2 binding to Cip1/p21 and Kip1/p27; quantification of bands showed 1.4- and 2.6-fold increases, respectively. No effect of silibinin, however, was observed on CDK4 and CDK6 binding to either Cip1/p21 or Kip1/p27 (Fig. 5 *F* and *G*). Together, these results clearly indicate that whereas the resultant effect of silibinin was a G_1 arrest, its causes were different in terms of molecular mechanisms at early G_1 and late G_1- to S-phase transition.

Silibinin Does Not Induce Apoptosis and Modulation of p53 and bcl2 Protein Levels in LNCaP Cells. Based on observed effects of silibinin, we next assessed whether silibinin causes apoptotic death of LNCaP cells. The 25-, 50-, and 75-μg/ml doses of silibinin for 24 and 48 hr did not result in apoptosis as evidenced by a lack of DNA fragmentation (Fig. 6*A*) and a lack of poly (ADP ribose) polymerase (PARP) cleavage that otherwise was clearly evident in a paclitaxel-treated sample used as a positive control (Fig. 6*B*). Because p53 and bcl2 are considered to be crucial in apoptosis (24), we also assessed their levels after

silibinin treatment. As shown in Fig. 6 *C* and *D*, silibinin also did not result in any change in p53 and bcl2 expression; however, paclitaxel (a positive control) showed a clear phosphorylation of bcl2 (Fig. 6*D*), a process associated with inactivation of bcl2 that causes apoptosis in LNCaP cells (25). Paclitaxel also showed clear morphological changes suggestive of apoptosis (data not shown), but no such effect was evident with silibinin, and, in fact, cells started showing differentiation (Fig. 7). These results suggest that silibinin-induced G_1 arrest in LNCaP cells does not lead to an apoptotic cell death.

Silibinin Induces Neuroendocrine Differentiation and Expression of K8 & K18 and Chromogranin A in LNCaP Cells. LNCaP cells treated with silibinin manifested unique morphologic changes. Compared with cells growing in 10% FBS as piled up layers attached loosely to the surface, cells treated with silibinin primarily were monolayer and attached firmly to the surface with better anchoring (Fig. 7 *A* vs. *B*). Significant changes in morphology also were observed with silibinin as cells became elongated with prominent dendrite-like cytoplasmic extensions where some of the dendrite-like extensions were connected to each other among neighboring cells (Fig. 7*B*). These morphological changes were similar to that of neuroendocrine morphology, suggesting that silibinin induces neuroendocrine differentiation of LNCaP cells (Fig. 7*B*). LNCaP cells grown in 10% cFBS also showed similar morphological changes (Fig. 7*C*), which were reversed to normal growth morphology by 1 nM DHT (Fig. 7*D*); the addition of silibinin reversed DHT-stimulated growth effect and induced similar neuroendocrine morphology in LNCaP cells (Fig. 7*E*). Silibinin treatment of cells grown in 10% FBS (or cells grown in 10% cFBS + 1 nM DHT; data not shown) also resulted in a significant induction of K8 & K18 and chromogranin A expression under identical conditions that showed neuroendocrine differentiation (Fig. 7*F*). The observed increases in K8 & K18 and chromogranin A by silibinin were optimum at both 24 and 48 hr (Fig. 7*F*). K8 & K18 have been shown to be markers of prostate tissue differentiation, and both K8 & K18 and chromogranin A are induced during differentiation of LNCaP cells with similar neuroendocrine-morphological changes (26, 27). These data suggest that silibinin induces neuroendocrine differentiation of LNCaP cells after G_1 arrest in cell cycle progression coupled with inhibition of growth-stimulatory pathways mediated by both serum as well as androgen.

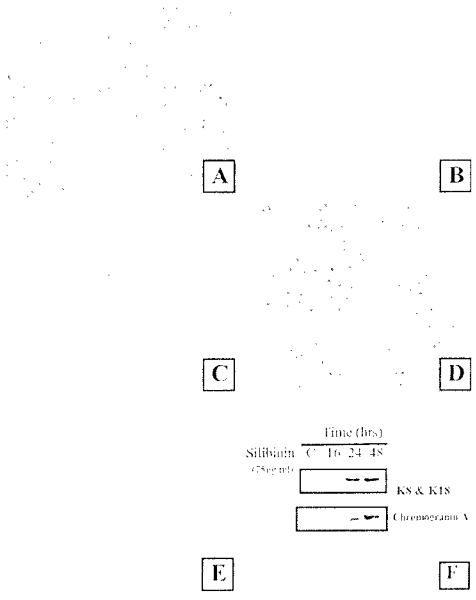

FIG. 7. Silibinin induces neuroendocrine differentiation and expression of K8 & K18 and chromogranin A in LNCaP cells. Morphology of LNCaP cells grown in 10% serum (*A*); 10% serum + 50 μg/ml silibinin (*B*); 10% cFBS (*C*); 10% cFBS + 1 nM DHT (*D*); and 10% cFBS + 1 nM DHT + 50 μg/ml of silibinin (*E*). The data are at 5 days of cultures; silibinin was added at day 3. The phase-contrast photography was done at ×200 magnification as described in *Materials and Methods*. (*F*) Stimulatory effect of silibinin on K8 & K18 and chromogranin A levels. Cells were treated with 75 μg/ml of silibinin for the indicated time; C, control cells treated with ethanol for 48 hr. Cell lysates were prepared, and levels of K8 & K18 (*Upper*) and chromogranin A (*Lower*) were determined as described in *Materials and Methods*. The data shown are representative of three independent experiments with similar findings.

DISCUSSION

LNCaP cells are one of the best *in vitro* models for human PCA studies because they possess an aneuploid male karyotype, produce PSA, and express a high-affinity mutant AR (28). These cells are responsive to androgenic stimulation and form tumors in nude mice (29). Because reduction in serum PSA levels has been proposed as an endpoint biomarker for hormone-refractory human PCA intervention (9–11), our results showing that silibinin significantly decreases both intracellular and secreted levels of PSA in androgen-dependent human PCA LNCaP cells have useful implications for human PCA intervention.

PSA is an abundant serine protease produced by prostate epithelial cells (30) and can cleave predominant seminal vesicle protein (31). PSA secretion by tumor cells into prostate stroma might augment cleavage of IGFBP3-IGF-1 and the activation of transforming growth factor β or other growth factors in extracellular matrix and then endow cancerous cells with a growth advantage leading to tumor progression (8). This hypothesis explains why PCA cells tend to diffusely infiltrate prostatic stroma rather than forming a localized tumor (8). Therefore, inhibition of PSA secretion may be an important strategy to prevent PCA progression. Here, we showed that a percentage decrease by silibinin in secreted PSA levels was comparable to intracellular PSA, suggesting that a decrease in PSA secretion by silibinin may be due to its inhibitory effect on PSA protein expression in LNCaP cells. Because silibinin also inhibited DHT-induced PSA and cell growth, we suggest that silibinin may have a direct effect on AR-mediated PSA expression.

Mammalian cell growth and proliferation are mediated via cell cycle progression (22, 23). However, defects in cell cycle are one of the most common features of cancer cells, because they divide under conditions in which their normal counterparts do not (22, 23). Androgen is shown to regulate genes controlling cell cycle, and that abnormally activated AR activity (e.g., gain-of-function by mutations in AR) may malignantly stimulate cell growth (32). Therefore, agents that inhibit cell cycle progression of cancer cells could lead to a cell growth arrest. We provide convincing evidence that silibinin inhibits both serum- and androgen-stimulated LNCaP cell growth by inducing G_1 arrest. The results from molecular mechanism studies showed that G_1 arrest by silibinin involves a significant decrease in cyclin D1, CDK4, and CDK6, resulting in a marked decrease in their kinase activity, and a significant increase in Cip1/p21 and Kip1/p27 that leads to their increased binding with CDK2, resulting in a marked decrease in CDK2 and cyclin E kinase activity.

Cyclin D1 is involved in cell cycle during early G_1 phase (23). In controlled cell growth, association of cyclin D1 with CDK4 or CDK6 leads to phosphorylation of RB; hyperphosphorylated RB leads to its release from E_2F (33). The free E_2F then activates c-*myc*, resulting in cell proliferation by progression via G_1 (34). However, overexpression of cyclin D1 is associated with various cancers and tumor-derived cell lines, explaining their uncontrolled growth (35). One of the aspects of cyclin D1 overexpression in cells is a shorten G_1 phase, resulting in a more rapid entry into S phase and increased proliferation (35). Based on these and other studies (34–36), a significant decrease in protein levels of cyclin D1, CDK4, and CDK6 by silibinin suggests that silibinin should be a useful agent for the intervention of malignancies overexpressing cyclin D1, CDK4, and/or CDK6. The observed inhibitory effects of silibinin on cyclin D1, CDK4, and CDK6 in

LNCaP cells are of particular significance for the intervention of hormone-refractory PCA because cyclin D1 is strongly associated with androgen-stimulated growth of LNCaP cells (37). Cyclin D1 is also constitutively expressed in androgen-independent human PCA PC3 and DU145 cells, but it is significantly lower in LNCaP cells grown without serum (38). In a recent study, overexpression of cyclin D1 in LNCaP cells was shown to increase cell growth and tumorigenicity in nude mice (39). Consistently, we found that LNCaP cells grown in cFBS arrest mostly in G_1 phase, which is reversed by DHT. This finding suggests the involvement of androgen-mediated growth after the release of cells from G_1 arrest because of a significant decrease in cyclin D1 in the absence of androgen. Similarly, silibinin treatment of LNCaP cells grown in serum or cFBS + DHT also showed a G_1 arrest together with a decrease in serum- and androgen-stimulated PSA levels and cell growth inhibition. These results suggest that observed effects of silibinin are those mediated via AR in terms of PSA levels, cell growth, cell cycle progression, as well as modulation of cyclin D1 and associated CDKs. In support of this suggestion, we recently have shown that treatment of human PCA DU145 cells with silymarin does not involve alterations in cyclin D1 for G_1 arrest (40). More detailed studies are in progress to support the involvement of AR in the inhibitory effects of silibinin.

p53 is an important tumor-suppressor gene, and mutations in p53 are the most commonly observed genetic lesions in human tumors (41). In response to genotoxic stress, p53 induces Cip/p21, resulting in a G_1 arrest (42). However, activation of Cip1/p21 also occurs independent of p53 as observed by transforming growth factor β stimulation during differentiation or upon cellular senescence (43). In each case, up-regulation of Cip1/p21 correlated with an arrest in cell growth, suggesting that it plays a fundamental role in the decision fork between cell proliferation, differentiation, and death. For example, inhibition of Cip1/p21 expression through transfection of Cip1/p21 antisense oligonucleotides was shown to block growth factor-induced differentiation of SH-SY5Y neuroblastoma cells and resulted in their death (44). Cip1/p21 induction also is shown in a variety of cell differentiation, including myogenic, keratinocytic, promyelocytic (HL-60), and human melanoma cells (45–47); Kip1/p27 also has been reported to be involved in cell differentiation (48). Consistently, we observed that silibinin-caused induction of Cip1/p21 was p53-independent and that, together with resultant G_1 arrest, did not induce apoptosis in LNCaP cells. Because treatment of LNCaP cells with silibinin showed neuroendocrine differentiation like morphologic changes and increased K8 & K18 and chromogranin A levels, induction of both Cip1/p21 and Kip1/p27 is likely to be involved with cell cycle exit that is associated with differentiation.

Together, the central finding in the present study is that silibinin, an active constituent of milk thistle, inhibits both serum- and androgen-stimulated PSA protein levels in LNCaP cells concomitant with cell growth inhibition via a G_1 arrest in cell cycle progression. The silibinin-treated LNCaP cells that are unable to grow follow a differentiation pathway as evidenced by neuroendocrine-like morphology, elevated prostate tissue-differentiation markers K8 & K18 and chromogranin A, and altered cell cycle-regulatory molecules. More detailed mechanistic studies are in progress to identify and define the effect of silibinin on the growth-stimulatory signals in hormone-refractory prostate carcinoma cells at molecular levels and to assess the inhibitory effect of silibinin on human PCA tumor xenograft growth in nude mice. In summary, however, based on the present findings, we conclude that silibinin has strong potential to be developed as an antiproliferative differentiating agent for the intervention of hormone-refractory human prostate cancer.

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Appendix 3

MOLECULAR CARCINOGENESIS 28:129–138 (2000)

FAST TRACK

Anticarcinogenic Effect of a Polyphenolic Fraction Isolated From Grape Seeds in Human Prostate Carcinoma DU145 Cells: Modulation of Mitogenic Signaling and Cell-Cycle Regulators and Induction of G1 Arrest and Apoptosis

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There is an increasing interest in identifying potent cancer preventive and therapeutic agents against prostate cancer (PCA). In a recent study, we showed that a polyphenolic fraction isolated from grape seeds (hereafter referred to as GSP) that is substantially rich in antioxidant procyanidins exerts exceptionally high preventive effects against tumorigenesis in a murine skin model. In the present study, we investigated the anticarcinogenic effect of GSP against PCA by employing DU145 human prostate carcinoma cells. GSP treatment (10–100 μg/mL doses for 2–6 d) of cells resulted in a highly significant ($P < 0.01$–0.001) inhibition of cell growth in both dose- and time-dependent manner. Compared with the vehicle, 2 d of GSP treatment resulted in 27, 39, and 76% growth inhibition at 50, 75, and 100 μg/mL doses, respectively, whereas 28–97% and 12–98% inhibition was evident at 10–100 μg/mL doses of GSP after 4 and 6 d of treatment, respectively. These doses of GSP also resulted in dose- and time-dependent cell death (6–50%, $P < 0.1$–0.001) that was later characterized as apoptotic death. In molecular mechanistic studies, treatment of DU145 cells with GSP at 25–75 μg/mL doses for 24, 48, and 72 h resulted in 77–88%, 65–93%, and 38–98% reduction, respectively ($P < 0.001$), in phospho–extracellular signal-regulated protein kinase (ERK) 1 and 78%, 19–76%, and 63–71% reduction ($P < 0.1$–0.001) in phospho-ERK2 levels, respectively. In other studies, similar doses of GSP showed up to 1.9-fold increases in Cip1/p21 and a significant ($P < 0.001$) decrease in cyclin-dependent kinase (CDK) 4 (up to 90% decrease), CDK2 (up to 50% decrease), and cyclin E (up to 60% decrease). GSP treatment of DU145 cells also resulted in a significant ($P < 0.001$) G1 arrest in cell-cycle progression in a dose-dependent manner. The growth-inhibitory and cell-death effects of GSP were also observed in another human PCA line, LNCaP. Together, these results suggest that GSP may exert strong anticarcinogenic effect against PCA and that this effect possibly involves modulation of mitogenic signaling and cell-cycle regulators and induction of G1 arrest, cell-growth inhibition, and apoptotic death. *Mol. Carcinog. 28:129–138, 2000.*

Key words: grape seeds; procyanidins; prostate cancer; mitogen-activated protein kinases; cell cycle; apoptosis

INTRODUCTION

Prostate cancer (PCA) is the most common non-skin malignancy and the second leading cause of cancer death in men in the United States [1]. One approach to control PCA is chemopreventive intervention, a means of cancer control whereby the disease is prevented, slowed, or reversed by the administration of one or a combination of naturally occurring or synthetic compounds. Several studies have shown that microchemicals present in the diet and in several herbs and plants are the most desirable class of agents for the prevention and/or intervention of various cancers reviewed in 2–7. Among these chemicals, polyphenolic antioxidants have received increasing attention in recent years [8–10]. One such naturally occurring antioxidant is a polyphenolic fraction isolated from grape seeds that is rich in procyanidins.

Grapes (*Vitis vinifera*) are one of the most widely consumed fruits in the world. Grapes are rich in polyphenols, and approximately 60–70% of grape

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Abbreviations: PCA, prostate cancer; GSP, a polyphenolic fraction isolated from grape seeds; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; CDK, cyclin-dependent kinase; ECL, enhanced chemiluminescence; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PI, propidium iodide; PS, phosphatidylserine; CDKI, cyclin-dependent kinase inhibitor.

polyphenols exist in grape seeds [11,12]. The combined name for grape seed polyphenols is procyanidins (or proanthocyanidins). Commercial preparations of grape seed polyphenols are marketed in United States as "grape seed extract" (GSP), with 95% standardized procyanidins as a dietary supplement because of its health benefits, in particular the very strong antioxidant activity of procyanidins. In addition to grape seed, procyanidins are a diverse group of polyphenolics widely distributed in fruits and vegetables [13]. Recent studies have shown that procyanidins possess anti-inflammatory, antiarthritic, and antiallergic activities and prevent heart diseases and skin aging [14]. GSP has been shown to exert a much stronger oxygen-free radical scavenging effect than vitamins C and E [15,16]. Wine consumption has been reported to have many beneficial health effects; wine may also be a source of procyanidins [17,18]. Recent cell culture studies have shown that treatment of human breast carcinoma MCF-7, human lung cancer A-427, and human gastric adenocarcinoma CRL-1739 cells with commercial grape seed proanthocyanidin extract results in a dose- and time-dependent inhibition of cell growth [19]. The agent, however, is not effective on K562 chronic myelogenous leukemic cells and enhances the growth and viability of normal human gastric mucosal cells and normal J774A.1 murine macrophage cells [19]. With regard to its anticarcinogenic effects in animal models, oral feeding of 1% (w/w) GSP in the diet has been shown to inhibit *APC* mutation–associated intestinal adenoma formation in MIN mice [20]. More recent studies [21,22] have shown that topical application of GSP significantly prevents tumor promotion in mouse skin. Regarding epidemiology, a case-control study showed that increased consumption of grapes is associated with reduced cancer risk [23].

As in other epithelial malignancies such as breast and lung, the autocrine and paracrine growth factor receptor interactions (e.g., transforming growth factor α– or epidermal growth factor–epidermal growth factor receptor, insulinlike growth factor 1–insulinlike growth factor 1 receptor) are the major contributors to uncontrolled prostate cancer cell growth (reviewed in [24]). The activation of these and other signaling cascades ultimately activates mitogen-activated protein kinases (MAPKs) that, after their translocation to the nucleus, activate transcription factors and command cell-cycle regulatory molecules for cell growth, proliferation, and differentiation [25–30]. Several studies have shown that MAPK extracellular signal-regulated protein kinases (ERKs) 1 and 2 are constitutively active in human prostate carcinomas and derived cell lines and possibly play a causative role in the progression of this malignancy from an androgen-dependent phenotype to an advanced and androgen-independent metastatic disease [31,32].

Based on these studies, we rationalized that GSP and the procyanidins present therein could also be a potent class of anticarcinogenic agents against human PCA. In this communication, for the first time we report that GSP exerted strong anticarcinogenic activity against DU145 androgen-independent human prostate carcinoma cells and that this effect was associated with (i) inhibition of constitutive activation of MAPK/ERK1/2, (ii) alterations in cell-cycle regulators, and (iii) G1 arrest, cell-growth inhibition, and apoptotic death. The cell-growth inhibitory and apoptotic effects of GSP were also evident in LNCaP androgen-dependent human prostate carcinoma cells.

MATERIALS AND METHODS

Cell Lines and Other Reagents

DU145 and LNCaP cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI-1640 with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. Anti-Cip1/p21 antibody was obtained from Calbiochem (Cambridge, MA) and anti–cyclin-dependent kinase (CDK) 4 antibody was obtained from Neomarkers, Inc. (Fremont, CA). Antibodies to cyclin D1, cyclin E, and CDK2 and rabbit anti–mouse immunoglobulin and goat anti–rabbit immunoglobulin horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Phospho- (and regular) MAPK/ERK1/2 antibodies were obtained from New England Biolabs (Beverly, MA). Enhanced chemiluminescence (ECL) detection system was obtained from Amersham (Arlington Heights, IL).

The GSP preparation used was obtained from Traco Labs (Champaign, IL). For all the studies, GSP was dissolved in dimethyl sulfoxide (DMSO) as a 20 mg/mL stock solution and diluted as desired directly in the medium. Unless specified otherwise, the final concentration of DMSO in culture medium during GSP treatment did not exceed 0.5% (v/v); therefore, the same concentration of DMSO was present in control dishes.

Cell Growth and Death Assays

DU145 and LNCaP cells were plated at 5000 cells/cm^2 density in 35-mm dishes under the culture conditions described earlier. After 24 h (~30–35% cell confluency), cells were fed with fresh medium and were treated with either DMSO alone or different concentrations (10–100 μg/mL) of GSP. The cultures were fed with fresh medium with or without the same concentrations of GSP every alternate day to the end of the experiment. Each

treatment and time point had three to four plates. After 2, 4, and 6 d of these treatments, both attached and floating cells were collected by trypsinization and counted with a hemocytometer. Trypan blue dye exclusion was used to determine viable and dead cells. This experiment was repeated two more times. The representative data shown in this report were reproducible in three independent experiments.

GSP Treatment of Cells for MAPK/ERK1/2 and Cell-Cycle Molecule Studies

DU145 cells were grown in 100-mm dishes, as described earlier, and at 70% confluency were treated with either DMSO alone or different concentrations (25–75 μg/mL) of GSP. After 24, 48, and 72 h of these treatments, medium was aspirated and cells were washed two times with cold phosphate-buffered saline (PBS). To each dish was added 0.5 mL of lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethyl sulfonyl fluoride, 0.5% NP-40, 1 μg/mL pepstatin, and 0.2 U/mL aprotinin). After 15 min in lysis buffer at 4°C, the cell lysate was scraped from the plate, collected in microfuge tubes, and left on ice for an additional 15 min. The lysates were cleared by centrifugation for 5 min in a table-top centrifuge at 4°C; the clear supernatants were collected and protein concentration was determined.

For western immunoblotting, 40–100 μg of protein lysate per sample was denatured with 2× sample buffer, samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels, and separated proteins were transferred on to membrane. The levels of phospho- and regular MAPK/ERK1/2, Cip1/p21, CDK4, cyclin D1, CDK2, and cyclin E were determined with specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system, as described in detail elsewhere [24]. Autoradiograms of the western immunoblots were scanned with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using the Scanimage Program (National Institutes of Health, Bethesda, MD). In case of ERK1/2 phosphorylation studies, the densitometric analysis data for phospho-ERK1/2 blots were corrected for loading with the density of ERK1/2 blots. The data shown are mean ± standard error of four independent experiments. In the case of cell-cycle regulatory molecules, the densitometric data shown in each case are the mean ± standard error of four to six independent experiments.

Cell-Cycle Analysis

DU145 cells at 70% confluency were treated either with DMSO alone or different concentrations (25–75 μg/mL) of GSP. After 24, 48, and 72 h of these treatments, medium was aspirated, cells were quickly washed two times with cold PBS and trypsinized, and cell pellets were collected. Approximately 0.5×10^6 cells in 0.5 mL of saponin/propidium iodide (PI) solution (0.3% saponin [w/v], 25 μg/mL PI [w/v], 0.1 mM EDTA, and 10 μg/mL RNase [w/v] in PBS) were left at 4°C for 24 h in the dark. Cell-cycle distribution was then analyzed by flow cytometry by using the FACS Analysis Core Services of the University of Colorado Cancer Center, Denver. This experiment was repeated at least two more times. The representative data shown in this article were reproducible in three independent experiments.

Apoptotic Death Assays

For qualitative assessment of apoptotic death, the DNA ladder assay was performed. Briefly, DU145 cells were grown in 100-mm dishes and at 70% confluency were treated with either DMSO alone or different concentrations (25–75 μg/mL) of GSP. After 48 and 72 h of these treatments, both floating and attached cells were collected, and the DNA ladder analysis was performed as described elsewhere [33]. To further substantiate the apoptotic death effects of GSP on DU145 cells and to quantify apoptosis, annexin V and PI staining of the cells was performed, followed by flow cytometry. In viable cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS to the external cellular environment [34]. The human anticoagulation annexin V is a 35- to 36-kD Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS. Annexin V labeled with a fluorophore or biotin can identify apoptotic cells by binding to PS exposed on the outer leaflet.

After treatment of cells with GSP at desired doses and treatment times, both floating and attached cells were collected and subjected to annexin V and PI staining by using Vybrant Apoptosis Assay Kit 2 (Molecular Probes, Inc., Eugene, OR) and following the step-by-step protocol provided by the vendor. The kit contains recombinant annexin V conjugated to fluorophores and the Alexa Fluro 488 dye, providing maximum sensitivity. Alexa Fluro 488 dye is an almost perfect spectral match to fluorescein, but it creates brighter and more photostable conjugates. In addition, the kit includes a ready-to-use solution of the red fluorescent PI, the nucleic acid binding dye. PI cannot permeate to live cells and apoptotic cells but stains necrotic cells with red

fluorescence, binding tightly to the nucleic acids in the cell. After staining of the cells with Alexa Fluro 488 annexin V and PI, flow cytometry was performed with a 488-nm line of an argon-ion laser for excitation. The apoptotic cells stained with annexin V showed green fluorescence, dead cells stained with both annexin V and PI showed red and green fluorescence, and live cells showed little or no fluorescence.

Statistical Analysis

As needed, Student's two-tailed t test was employed to assess the statistical significance of difference between the vehicle- and GSP-treated samples.

RESULTS

GSP Inhibited the Growth of DU145 Cells and Caused Their Death

GSP treatment of DU145 cells resulted in significant inhibition of their growth in a dose- and time-dependent manner. Compared with vehicle-treated controls, 2 d of GSP treatment at 10 and 25 μg/mL doses was not effective; in contrast, 50, 75, and 100 μg GSP/mL doses showed 27, 39, and 76% inhibition ($P < 0.001$), respectively (Figure 1A). A much stronger inhibitory effect of GSP on DU145 cell growth was evident after 4 and 6 d of treatment; all the doses showed a strong effect, accounting for 28–97% and 12–98% inhibition ($P < 0.01$–0.001), respectively (Figure 1A). In cell-death measurements, GSP showed both dose- and time-dependent death of DU145 cells (Figure 1B). Compared with the DMSO control showing 3–8% death during 2, 4, and 6 d, GSP treatment resulted in 7–12%, 8–30%, and 7–47% ($P < 0.1$–0.001) cell death after 2, 4, and 6 d of treatment at 10–100 μg/mL doses, respectively (Figure 1B).

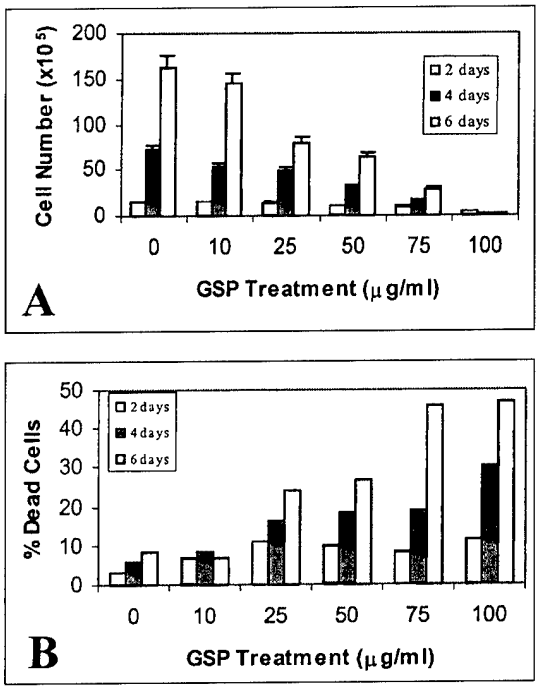

Figure 1. GSP inhibited the growth of DU145 cells. Cells were plated at 5000 cells/cm^2 in 35-mm dishes. After 24 h, cultures were treated with DMSO or GSP at the concentrations of 10–100 μg/mL of medium. After 2, 4, and 6 d of these treatments, both attached and floating cells were collected by trypsinization and counted with a hemocytometer. Trypan blue dye exclusion was used to determine viable and dead cells. The cell-growth data (A) and cell-death data (B) are presented as mean ± SE of four independent plates; each sample was counted in duplicate. This experiment was repeated two more times. The representative data shown in this figure were reproducible in three independent experiments.

GSP Inhibited Constitutive Activation of MAPK/ERK1/2 in DU145 Cells

The autocrine growth factor receptor feedback loop is well known for autonomous growth of DU145 cells. Consistent with this fact, these cells were also been shown to contain a constitutively active MAPK/ERK1/2 [24,31]. Based on our data showing that GSP significantly inhibited the growth of these cells, to identify the molecular targets of this effect, we first focused our attention on the effect of GSP on constitutive activation of MAPK/ERK1/2. As shown in Figure 2A, treatment of DU145 cells with GSP resulted in a moderate to highly significant inhibition of constitutive activation of ERK1/2. The densitometric analysis of blots showed that, compared with DMSO control, 24 h of GSP treatment at 25, 50, and 75 μg/mL doses resulted in 67, 88, and 88% decreases ($P < 0.001$) in phosphorylated ERK1 (Figure 2A, upper band; Figure 2C, the densitometric analysis data). Whereas 48 and 72 h of GSP treatment at the 25 μg/mL dose did not show an additional decrease, the 50 μg/mL dose resulted in 93% inhibition ($P < 0.001$) in phosphorylated ERK1 level; no further decrease was evident by increasing the dose to 75 μg/mL as 90% inhibition was observed at this dose (Figure 2A and C). In the case of ERK2 phosphorylation (lower band in Figure 2A), 24 h of treatment with GSP at the 25 μg/mL dose was ineffective and the 50 μg/mL dose showed only 20% decrease (Figure 2A and D). A stronger decrease, however, was evident at the 75 μg/mL dose of GSP, accounting for 78% inhibition ($P < 0.001$). Similar effects on ERK2 phosphorylation were also observed after 48 h of GSP treatment at these doses, however, an approximately 70% decrease ($P < 0.001$) was evident at all the three doses of GSP after 72 h of treatment (Figure 2A and D).

GSP Induced Alterations in Cell-Cycle Regulators in DU145 Cells

Eukaryotic cell growth, proliferation, differentiation, and/or apoptosis are mediated by the cell-cycle progression that is governed by mitogenic signaling reviewed in 35–39. However, cancer cells often display defects in the genes that govern the cellular

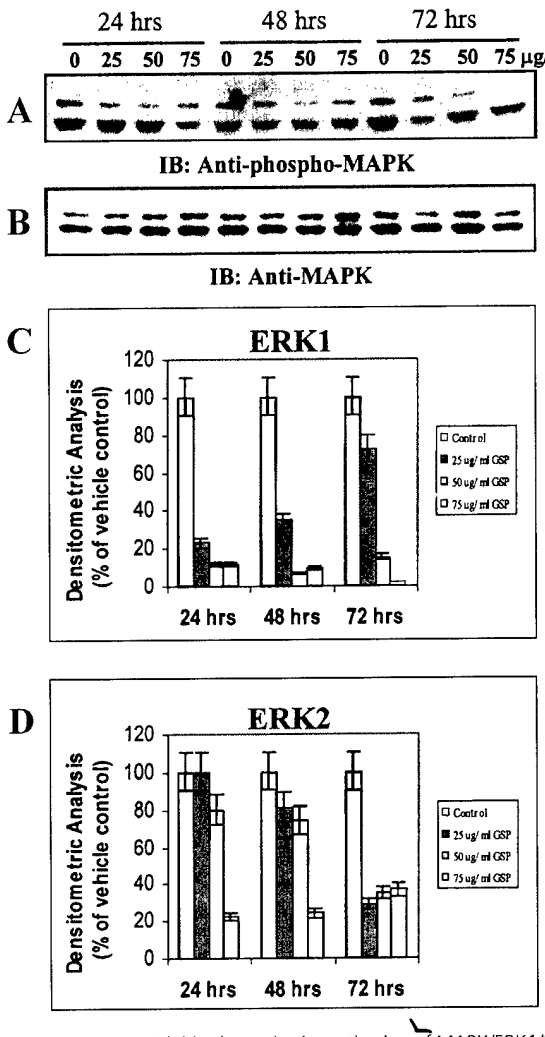

Figure 2. GSP inhibited constitutive activation of MAPK/ERK1/2 in DU145 cells. Cells were cultured in Dulbecco's minimum essential medium with 10% serum; at 70% confluency, cells were treated with either DMSO alone or 25, 50, and 75 μg/mL concentrations of GSP for 24, 48, and 72 h. Cell lysates were prepared and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western blotting, as described in Materials and Methods. The membranes were probed with anti–phospho-ERK1/2 and regular ERK1/2 antibodies and then with peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was performed with the ECL detection system. (A) Phosphorylation of ERK1/2. (B) Total levels of ERK1/2. Treatments are as labeled in the figure. IB, western immunoblot. (C and D) The densitometric analysis data for phospho-ERK1 (C) and phospho-ERK2 (D) blots were corrected for loading with the density of ERK1/2 blots. The data shown are mean ± SE of four independent experiments.

responses to interactions between growth factors and growth factor receptors; perturbations in cell-cycle regulation have been demonstrated to be one of the most common features of transformed cells [35–39]. These genetic alterations have been thought to be associated with the gain of function for uncontrolled growth due to enhanced expression of the growth factor–receptor autocrine loop, a lack of cyclin-dependent kinase inhibitor (CDKI) or loss of its function, and enhanced expression of CDKs and their catalytic subunit cyclins [35–39]. Accordingly, we assessed the effect of GSP on cell-cycle regulators in DU145 cells.

As shown in Figure 3A, exposure of cells to GSP resulted in a significant induction of Cip1/p21 protein levels. As quantitated by densitometric analysis of the immunoblots, treatment of cells with GSP at 25, 50, and 75 μg/mL doses for 24 h resulted in a 1.2–1.6-fold ($P < 0.01$–0.001) increase in Cip1/p21 protein (Figure 3A). A much more profound increase in Cip1/p21 was evident after 48 h of GSP treatment and accounted for 1.2-, 1.5-, and 1.9-fold ($P < 0.01$–0.001) induction at these doses, respectively (Figure 3A). No further increase in Cip1/p21 levels was evident after longer GSP treatment at identical doses; in fact, there was a loss of effect after 72 h of treatment (Figure 3A). In the studies assessing the effect of GSP on CDKs and cyclin levels, GSP treatment showed 30, 40, and 90% decreases ($P < 0.001$) in CDK4 after 24 h treatment; comparable results were also observed at 48 and 72 h (Figure 3B). Unlike CDK4, the decrease in CDK2 (~50%, $P < 0.001$) and cyclin E (up to 60%, $P < 0.001$) protein levels were evident only after 72 h of GSP treatment at higher doses (Figure 3C and 3D). GSP treatment of DU145 cells did not cause a decrease in cyclin D1 (data not shown).

GSP Induced G1 Arrest in Cell-Cycle Progression of DU145 Cells

Both cell growth and inhibition are mediated through cell-cycle progression [35–39]. Based on its effect on cell-cycle regulators, we next examined the effect of GSP on cell-cycle progression. As shown in Figure 4, compared with vehicle-treated control cells, a marked difference in cell-cycle progression was observed with GSP. Within 24 h of treatment, the 50 μg/mL dose showed 60.4% cells in G1 phase as opposed to 52% cells in the control cells (Figure 4). An increase in dose to 75 μg/mL of GSP resulted in a further increase in G1 population to 66.7% (Figure 4), and the cells mostly remained there at the later time points of 48 and 72 h (data not shown). The increase in G1 population by GSP at all the time points studied was accompanied by a decrease of the cells in both S and G2–M phases (Figure 4).

GSP Induced Apoptotic Death of DU145 Cells

Based on the data shown in Figures 1–4, we wondered about the ultimate fate of the GSP-treated cells. Do they undergo apoptotic cell death? Both qualitative and quantitative apoptotic cell-death studies were performed to answer this question. As shown in Figure 5A, compared with the vehicle control cells, treatment of DU145 cells with 25, 50, and 75 μg/mL doses of GSP for 48 h resulted in internucleosomal DNA fragmentation (lane 1 vs.

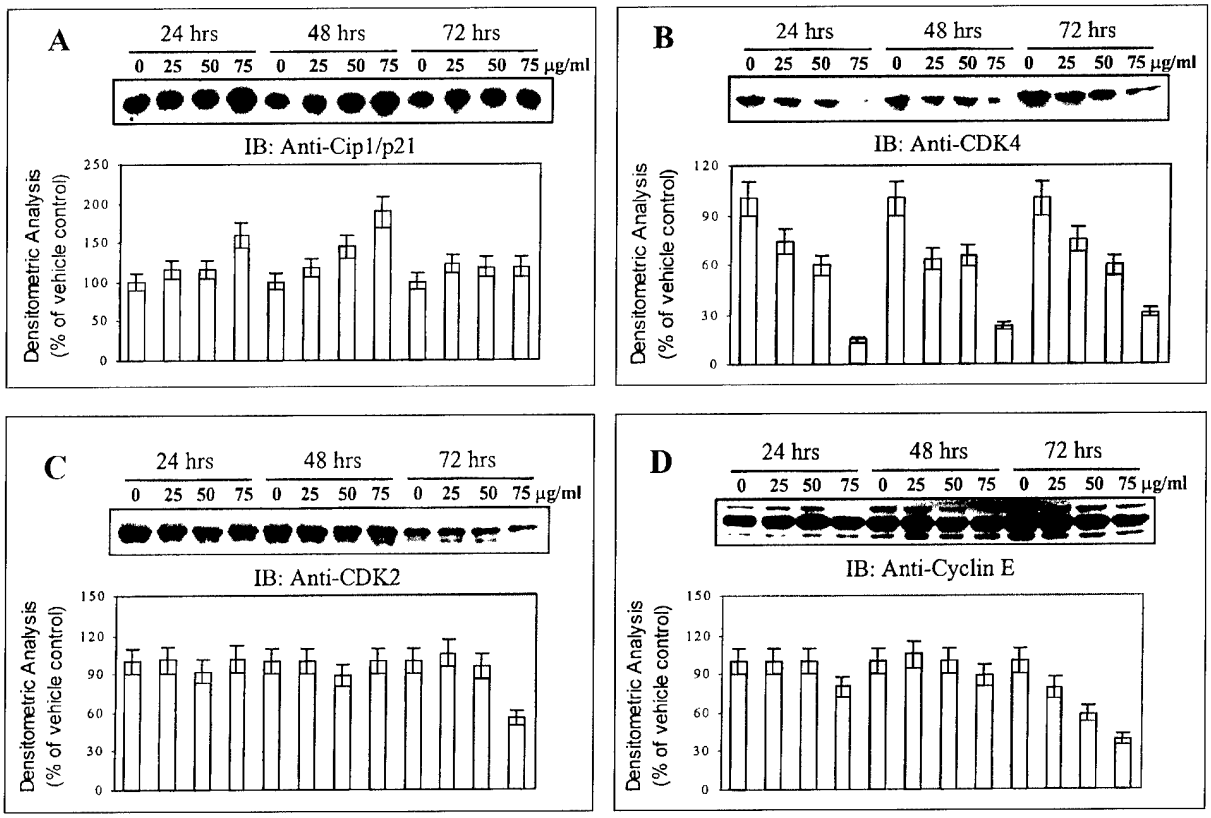

Figure 3. GSP induces alterations in cell cycle regulators in DU145 cells. Cells were cultured in Dulbecco's minimum essential medium with 10% serum; at 70% confluency, cells were treated with either DMSO alone or 25, 50, and 75 μg/mL concentrations of GSP for 24, 48, and 72 h. Cell lysates were prepared and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by western blotting, as described in Materials and Methods. The membranes were probed with anti-Cip1/p21 (A), anti-CDK4 (B), anti-CDK2 (C), and anti–cyclin E (D) antibodies and then with peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was performed with the ECL detection system. Treatments are as labeled in the figure. IB, western immunoblot. In each panel, the densitometric analysis data shown are presented as mean ± SE of four to six independent experiments. In panel D, the densitometric data shown are from the middle band.

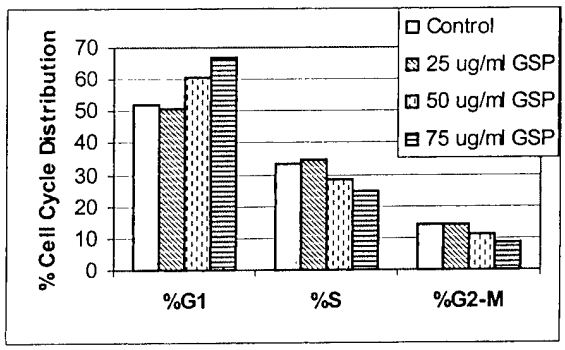

Figure 4. GSP induced G1 arrest in the cell-cycle progression of DU145 cells. Cells were cultured in Dulbecco's minimum essential medium with 10% serum; at 70% confluency, cells were treated with either DMSO alone or 25, 50, and 75 μg/mL concentrations of GSP for 24 h. After these treatments, cells were trypsinized and cell pellets were collected. Approximately 0.5×10^6 cells were labeled with PI using 0.5 mL of saponin/PI solution, at 4°C for 24 h in the dark, as described in Materials and Methods. Cell-cycle distribution was then analyzed by flow cytometry with the Becton Dickinson FACS System. The data shown are representative of three independent experiments (each done in duplicate) with less than 3% variation.

lanes 2–4, respectively). A stronger DNA fragmentation was evident at these doses after 72 h of GSP treatment (Figure 5A, lane 5 vs. lanes 6–8). The paclitaxel used as a positive control in these studies showed a DNA fragmentation pattern similar to that of GSP (Figure 5A, lane 9).

For the quantification of apoptotic cell death, annexin V and PI were used for staining [34]. As shown in Figure 5B, compared with the DMSO-treated vehicle control cells, which showed 7% annexin V–positive apoptotic cells, 24 h of GSP treatment at 25, 50, and 75 μg/mL doses resulted in 14, 15, and 18% ($P < 0.001$) apoptotic cells, respectively. After 48 h of treatment at these doses, 17, 28, and 27% ($P < 0.001$) annexin V–positive apoptotic cells were evident, versus 8% in the DMSO control cells (Figure 5B). A further increase in apoptotic cells was observed at the 25 and 50 μg/mL doses, accounting for 34 and 37% ($P < 0.001$) apoptotic cells, respectively; however, at the 75 μg/mL dose of GSP, there was a strong decrease in apoptotic cells

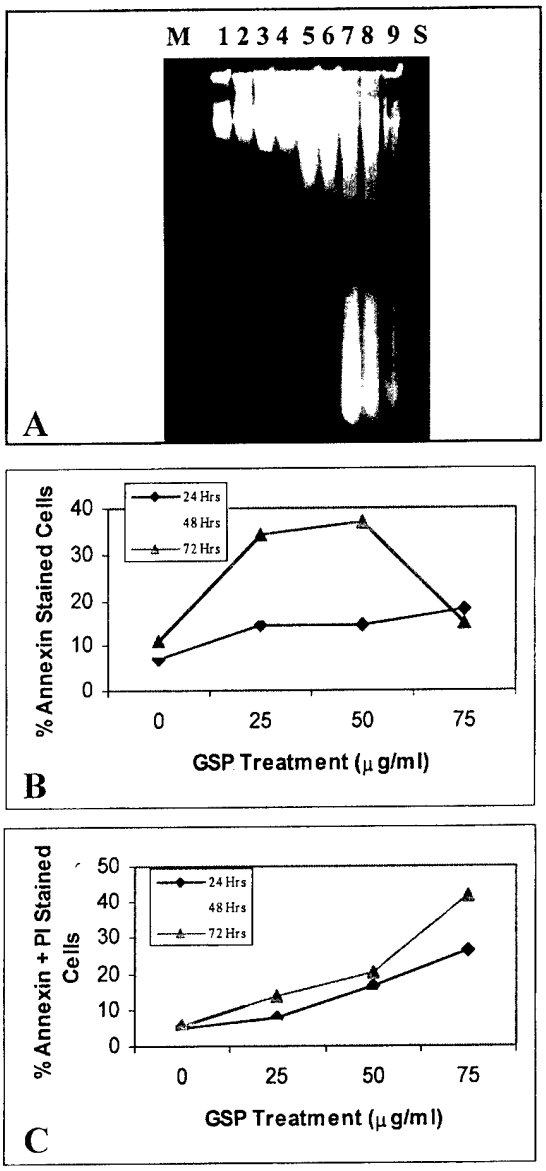

Figure 5. GSP induced apoptotic death of DU145 cells. (A) DU145 cells were grown in 100-mm dishes and, at 70% confluency, were treated with either DMSO alone or different concentrations (25–75 μg/mL) of GSP. After 48 and 72 h of these treatments, both floating and attached cells were collected, and the DNA ladder analysis was performed, as described in Materials and Methods. Lane M: DNA ladder marker. Lanes 1–4 and 5–8: 0, 25, 50, and 75 μg/mL doses of GSP for 48 and 72 h, respectively. Lane 9: 1 μM paclitaxel for 20 h as a positive control. S, standard sample provided in the kit. (B and C) Percentages of annexin V– and PI-stained cells, respectively. DU145 cells at 40–50% confluency were treated with DMSO alone or with different concentrations of GSP (25–75 μg/mL) in DMSO. After 24, 48, and 72 h of these treatments, adherent and cells that had detached from the monolayer were processed together. Cells were washed in PBS, stained with annexin V and PI, and analyzed by flow cytometry, as described in Materials and Methods. The data shown were reproducible in two independent experiments with less than 3% variation; each treatment had two independent plates.

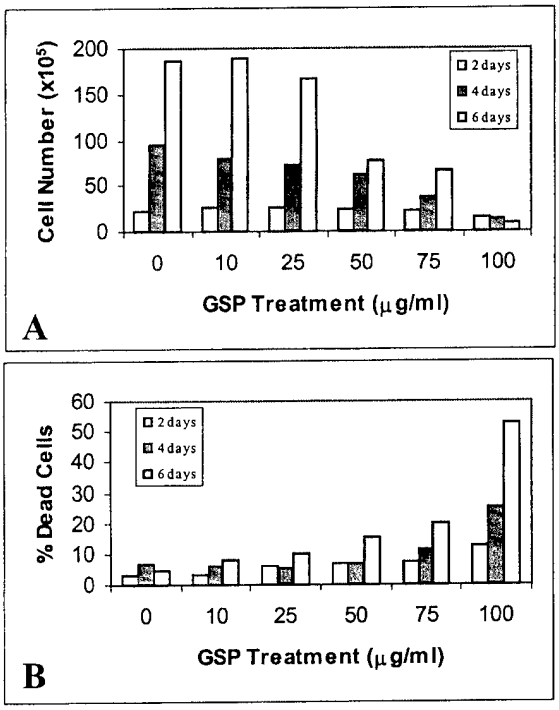

Figure 6. GSP inhibited the growth of LNCaP cells. Cells were plated at 5000 cells/cm^2 in 35-mm dishes. After 24 h, cultures were treated with DMSO or GSP at the concentration of 10–100 μg/mL of medium. After 2, 4, and 6 d of these treatments, both attached and floating cells were collected by trypsinization and counted with a hemocytometer. Trypan blue dye exclusion was used to determine viable and dead cells. The cell-growth data (A) and cell-death data (B) are presented as mean ± SE of four independent plates; each sample was counted in duplicate. This experiment was repeated two more times. The representative data shown in this figure were reproducible in three independent experiments.

that was possibly associated with necrotic death (Figure 5B and C). When the results were analyzed in terms of annexin V plus PI staining of the cells, only a dose-dependent effect of GSP was evident at all the time points studied (Figure 5C). In terms of time response, compared with the 24-h data, the 48-h results showed less effect; however, after 72 h of treatment, a further increase in staining was evident at all three doses (Figure 5C). Together, these results suggest that, possibly as an initial response, GSP induces apoptosis at low doses and shorter treatment duration. However, higher doses and/or longer treatment time may cause both apoptosis and necrosis.

GSP also Inhibited Growth and Induced Death of LNCaP Cells

To evaluate whether the observed effect of GSP on DU145 cells is specific or whether other human prostate carcinoma cells also respond similarly to treatment with GSP, we assessed its effect on the growth and death of androgen-dependent human prostate carcinoma LNCaP cells. As shown in Figure 6A, treatment of LNCaP cells with GSP under identical conditions resulted in both dose- and time-dependent growth inhibitions. Whereas 2 d of GSP treatment at 10–100 μg/mL doses did not show any considerable effects, 25–100 μg/mL doses

of GSP showed 23–86% and 10–96% inhibition ($P < 0.01$–0.001) after 4 and 6 d of treatments, respectively (Figure 6A). In terms of cell death, GSP treatment at these doses resulted in a strong (12%) to highly significant (up to 53% death, $P < 0.001$) LNCaP cell death (Figure 6B).

DISCUSSION

It has been suggested that PCA induction followed by development and metastasis are stepwise processes, where androgen (testosterone) plays a major role during the early stage of the malignancy reviewed in 40,41. Accordingly, androgen ablation therapy is extensively used to control human PCA [42]. However, within a few years with this treatment modality, PCA regrowth occurs that is androgen independent. At this advanced and androgen-independent stage, functional autocrine and paracrine growth factor–growth factor receptor interactions have been shown as causative factors for uncontrolled PCA growth and metastasis [43–49]. For example, aberrant and high expression of erbB family members and associated ligands have been shown with high frequency in prostatic intraepithelial neoplasia and in invasive PCA, both primary and metastatic [43–49]. In a classic signaling cascade, an activation of growth factor receptor ultimately leads to an activation of ERK1/2 as a mitogenic signal for cell growth [25–30]. Accordingly, one can argue that in advanced and androgen-independent PCA, in which an autocrine growth factor–receptor interaction leads to an autocrine feedback loop, ERK1/2 could be constitutively active as an ultimate contributor for uncontrolled growth. Indeed, persistent activation of ERK1/2 has been reported in DU145 androgen-independent human prostate carcinoma cells [31]. In another recent study, increased expression of activated ERK1/2 has been demonstrated to be associated with the progression of advanced and androgen-independent human PCA [32]. The results of these studies and the present data obtained with GSP showing a strong decrease in constitutive activation of ERK1 and ERK2 (at high doses only) are specifically important because they mimic the in vivo PCA situation where this mitogenic signaling is constitutively active. At this point, we emphasize that, to the best of our knowledge, this is the first study showing such a strong inhibition of constitutive ERK1/2 activation in advanced and androgen-independent human PCA DU145 cells by a dietary agent. Based on this result, more studies are needed to further explore whether this is a direct effect or occurs through an inhibition of an upstream signaling pathway involving receptor tyrosine kinases and other cytoplasmic signals.

Eukaryotic cell-cycle progression is regulated by a series of CDKs and cyclins at different phases. Whereas CDK4 and/or CDK6 complexed with cyclin D regulate early G1 transition, CDK2 coupled with cyclin E is involved in late G1-S– and early S-phase transitions [35–39]. Cell-cycle progression is regulated through several different CDK regulatory mechanisms [35–39]. Two major mechanisms for CDK regulation are (i) binding with its catalytic subunit cyclin followed by activation of CDK-cyclin complexes and (ii) binding with CDKIs followed by inactivation of CDK-cyclin complexes [33]. An alteration in the formation of these complexes could lead to (i) an increased cell growth and proliferation or (ii) a decreased cell growth and proliferation followed by differentiation and/or cell death by apoptosis [35–39]. In our studies, therefore, an increase in Cip1/p21 and a strong decrease in the protein levels of cell-cycle regulators associated with G1 phase concomitant with G1 arrest after GSP treatment of cells suggest that GSP could be a useful agent for the preventive intervention of PCA. More detailed studies, however, are needed to define the mechanisms involved with the induction of Cip1/p21 after GSP treatment of DU145 cells and the role increased Cip1/p21 plays in inhibiting the growth of these cells through G1 arrest that possibly leads to apoptotic and necrotic death of DU145 cells. Detailed studies are also needed to assess and define the effect of GSP on different apoptotic pathways that are possibly independent of Cip1/p21 involvement. Such studies are presently in progress.

In summary, the novel finding in the present study is the identification of the anticarcinogenic effect of GSP against advanced and androgen-independent prostate carcinoma DU145 cells and its association with an inhibition of constitutive activation of ERK1/2 and alterations in cell-cycle regulators and G1 arrest, cell-growth inhibition, and apoptotic death. Because GSP is sold commercially over the counter as a dietary supplement, the in vivo validation of its inhibitory effects against human prostate carcinoma tumor xenograft growth in nude mice could lead to the development of GSP as human prostate cancer preventive and/or interventive agent. We are currently in the process of starting these nude-mice studies.

ACKNOWLEDGMENTS

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Appendix 4

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Anti-Angiogenic Potential of a Cancer Chemopreventive Flavonoid Antioxidant, Silymarin: Inhibition of Key Attributes of Vascular Endothelial Cells and Angiogenic Cytokine Secretion by Cancer Epithelial Cells

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In recent studies, we have shown that silymarin, a naturally occurring flavonoid antioxidant, exhibits anti-cancer effects against several epithelial cancers. Here, we assessed its potential as an anti-angiogenic agent employing human umbilical vein endothelial cells (HUVEC) and human prostate and breast cancer epithelial cells. When sub-confluent HUVEC were treated for 48 h, adherent cell number decreased by 50 and 90% at 50 and 100 μg/ml doses, respectively. Apoptotic cell death principally accounted for cell loss at >50 μg/ml doses. In biochemical analysis, silymarin treatment of HUVEC for 6 h resulted in a concentration-dependent decrease in the secretion and cellular content of matrix metalloproteinase (MMP)-2/gelatinase A. Silymarin also inhibited HUVEC tube formation (*in vitro* capillary differentiation) on a reconstituted extracellular matrix, Matrigel. In other studies, 5 to 6 h exposure of DU145 prostate, and MCF-7 and MDA-MB-468 breast cancer cells to silymarin resulted in a dose-dependent decrease in the secreted vascular endothelial growth factor (VEGF) level in conditioned media without any visible change in cell morphology. The inhibitory effect of silymarin on VEGF secretion occurred as early as 1 h. These observations indicate a rapid inhibitory action of silymarin on the secretion of this primary angiogenic cytokine by cancer epithelial cells. Taken together, the results of this study support the hypothesis that silymarin possesses an anti-angiogenic potential that may critically contribute to its cancer chemopreventive efficacy. © 2000 Academic Press

Key Words: **silymarin; HUVEC; MMP-2/gelatinase A; *in vitro* capillary differentiation; vascular endothelial growth factor; angiogenesis switch.**

It is now well established that angiogenesis, that is, the growth of capillary vessels from existing blood vessels, is obligatory for the growth and progression of solid cancers (1–3). During solid cancer genesis, initiated cells undergo clonal expansion in an avascular state when the expanding lesions are small enough to take in nutrients and to expel metabolic wastes by diffusion. However diffusion is not sufficient to support continued growth of the lesion beyond a certain physical size (estimated ~2 mm diameter) because the expanding lesions consume nutrients at a rate proportional to their volume whereas the supply of nutrients is delivered at a rate proportional to their surface area (4, 5). In order for avascular lesions to progress beyond the size limit imposed by simple diffusion, they must turn on their angiogenic switch to form a neovasculature. Angiogenesis critically depends on several conditions such as the endothelial cells must proliferate to provide the necessary number of cells for the growing vessels, the activated endothelial cells must secrete matrix metalloproteinases (MMP) required to break down surrounding tissue matrix and the endothelial cells must be capable of movement/migration. In addition, the angiogenic stimuli (for example, hypoxia and production of angiogenic cytokines such as vascular endothelial growth factor [VEGF]) must be sustained. Because of the critical dependence of tumor growth and metastasis on angiogenesis, therapeutic strategies have been developed targeting various aspects of the angiogenic processes, many with promising results. Cancer chemoprevention aims to block or reduce the occurrence or progression of human malignancies by the chronic administration of naturally occurring or synthetic chemical agents. Chemoprevention can be most effective on early lesions, the fate and growth of which are likely to be more critically dependent on angiogenesis. Since the vascular endothelial cells constitute the first line of exposure to blood-borne agents, it is plausible that cancer chemopreventive

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activity of many agents may be attributable, at least in part, to anti-angiogenic properties through an inhibition of one or more of the angiogenic responses of the endothelial cells.

Fruits, vegetables, tea as well as many medicinal herbs and plants have been shown to be rich sources of phytochemicals with chemoprevention potential for some kinds of human cancer (6–9). Naturally occurring polyphenolic antioxidants are among these phytochemicals that have received increasing attention in recent years (6–9). Silymarin is a polyphenolic flavonoid antioxidant isolated from milk thistle (*Silybum marianum (L.) Gaertn*) and is used clinically as a liver detoxicant for almost three decades (10, 11). Several studies in recent past have shown anti-carcinogenic effects of silymarin in short-term bioassays (12–14). More recently, we have shown the cancer preventive efficacy of silymarin in several mouse skin tumorigenesis models (15–19), and its anti-cancer potential for human breast, prostate and cervical cancers (20–24).

Whereas all the mechanistic studies done with silymarin in recent years have focused on the cancer epithelial cells as the targets, the present study was conducted to explore potential inhibitory effects of silymarin on key parameters critical for tumor angiogenesis. In this paper, we report that silymarin treatment of human umbilical vein endothelial cells (HUVEC) inhibits their growth and survival, the secretion and expression of matrix metalloproteinases (MMPs) and capillary tube formation (*in vitro* angiogenesis). In addition, we report a rapid inhibitory action of silymarin on the secretion of a primary angiogenic cytokine VEGF by human prostate and breast cancer epithelial cells. Together, these results support an anti-angiogenic activity of silymarin that may contribute critically to its cancer chemopreventive potential.

MATERIALS AND METHODS

Chemicals and reagents. Silymarin, bovine endothelial cell growth supplement (ECGS) and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). Matrigel was purchased from Becton-Dickinson Labware (Bedford, MA). VEGF ELISA kit was purchased from R&D Systems (Minneapolis, MN).

Cell lines and cell culture. HUVEC cells, DU145 prostate cancer cells, and MCF-7 and MDA-MB-468 breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). HUVEC were propagated in F12K medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml of heparin (Sigma Chemical Co., St. Louis, MO) and 30 μg/ml of bovine endothelial cell growth supplement (ECGS) (Sigma Chemical Co.) as described previously (25). DU145 cells were cultured in RPMI1640 medium supplemented with 10% FBS. MCF-7 and MDA-MB-468 breast cancer cells were cultured in DMEM medium supplemented with 10% FBS and 2 mM L-glutamine.

HUVEC growth/survival. Cells were seeded into 6-well plates for 24–48 h to reach ~50% confluence. Fresh medium was replaced and silymarin was added from 100× stock solutions prepared in DMSO/ethanol (20:80). In all the studies, the selection of silymarin doses was based on our earlier studies showing anti-proliferative and differentiation-inducing effects in several human epithelial carcinoma cells (20–24). Morphological responses were monitored over time under a phase contrast microscope. Adherent cells after 48 h of treatment were fixed in 1% glutaraldehyde and stained. The cell number was counted under 100× magnification for 5 random fields for each condition. The experiment was repeated at least once.

Zymogram analysis for MMP-2. HUVEC were grown in 6-well plates in complete medium for 24–48 h to near confluence. The cells were washed two times with PBS to remove spent medium and fed serum-free medium supplemented with 100 μg ECGS/ml and treated with silymarin for 6 h (a time frame that did not result in any visible morphological changes). Conditioned medium and cell lysate (prepared in 1% Triton X-100, 0.5 M Tris-HCl, pH 7.6, 200 mM NaCl) were analyzed for gelatinolytic activities on substrate gels as we previously described (25). The gels were digitized with a transmission scanner and band intensity (on inverted images) was quantified using the UN-SCAN-IT gel scanner software (Silk Scientific, Inc. Orem, UT). As a comparison for the efficacy of silymarin to inhibit HUVEC MMP-2, curcumin, a polyphenolic component of the food flavor turmeric, was included in some experiments. Curcumin has been reported to potently inhibit MMP-2 expression and tube formation in this model (26).

Capillary tube formation on Matrigel (in vitro angiogenesis). The method of Kubota *et al.* was used (27). When seeded on Matrigel, a reconstituted extracellular matrix preparation of EHS mouse sarcoma, vascular endothelial cells undergo rapid *in vitro* differentiation into capillary like structures (27), providing a simple assay for assessing impact of agents on endothelial differentiation process which requires cell-matrix interaction, intercellular communication as well as cell motility. To examine the effect of silymarin on this process, HUVEC were treated in two ways in relationship to the time frame of cell seeding onto the Matrigel. (A) Silymarin simultaneous with cell seeding: Twenty-four-well cell culture plates were coated with 0.3 ml of Matrigel and allowed to solidify at 37°C for 1 h. Then 0.5 ml medium was added to each well and silymarin was added at 2 times of the desired concentrations. HUVEC were trypsinized and 20,000 or 40,000 cells were added per well in 0.5 ml medium. Tube formation was observed periodically over time under a phase contrast microscope. Representative Polaroid pictures were taken at 6 or 17 h. (B) Treatment of preformed tubes: HUVEC were seeded onto Matrigel for 6 h to form rudimentary tubes, then the medium was replaced and silymarin was added. Tube morphology was observed over time and representative Polaroid pictures were taken at 20 h after the initiation of silymarin treatment. Curcumin was included in some experiments as a comparison for the efficacy of silymarin to inhibit tube formation. The experiments were repeated twice.

VEGF secretion and expression in cancer epithelial cells. In dose-response experiments, DU145 prostate cancer cells and MCF-7 (estrogen dependent) and MDA-MB-468 (estrogen independent) breast cancer cells were grown in T25 flasks in complete medium until confluence (~48 h). The spent medium was removed, and cells were washed 3× with PBS. Cells were treated in serum-free medium with increasing concentrations of silymarin. Conditioned media and cell lysates were analyzed for VEGF protein content by an ELISA kit as per manufacturer's instructions (R&D Systems, Minneapolis, MN). In time course experiments, confluent DU145 or MDA-MB-468 cells were treated in serum-free media with solvent vehicle (DMSO/ethanol), 50 or 100 μg/ml silymarin. Serial 1-ml aliquots were taken of the culture media for VEGF ELISA. Each sample was measured in triplicate. Experiments were repeated at least once.

RESULTS

HUVEC growth and survival. As shown in Fig. 1 (A–D), treatment with silymarin for 48 h led to a

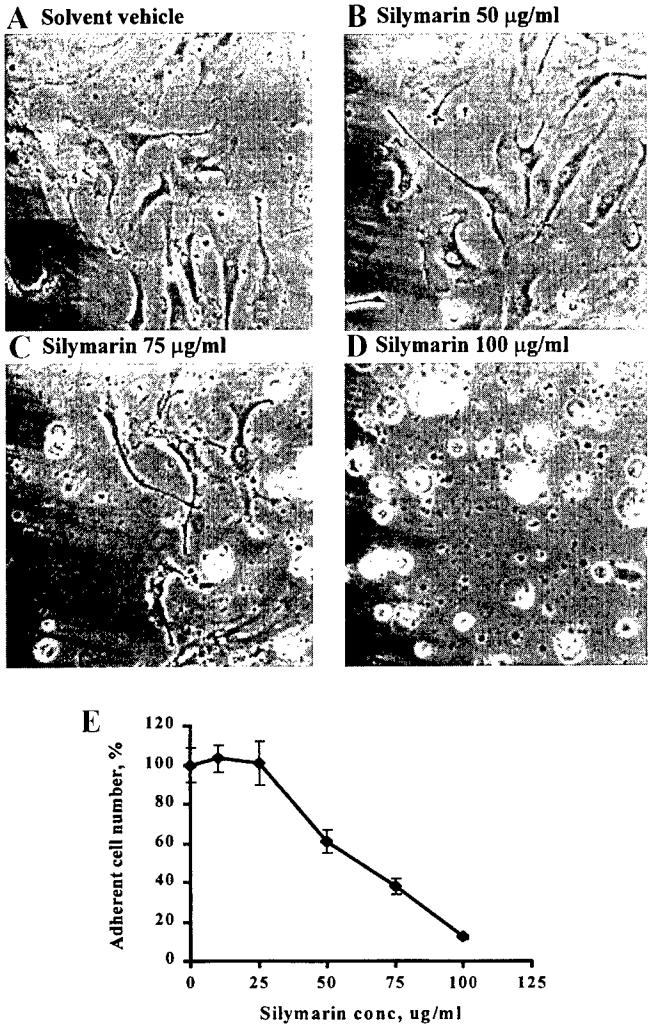

FIG. 1. Effect of silymarin treatment on HUVEC growth and survival. (A–D) Representative phase-contrast photomicrographs of HUVEC at 48 h after treatment was initiated with DMSO/ethanol vehicle (A), 50 (B), 75 (C), and 100 (D) μg/ml of silymarin. Most floaters showed typical apoptotic morphology such as cell retraction, condensation, and fragmentation into apoptotic bodies. (E) Adherent cell number as a function of initial silymarin treatment concentration. Each data point represented the mean ± SEM of the adherent cells in 5 randomly chosen fields.

concentration-dependent decrease of cells remaining adherent to the culture vessel and an increase of detached floaters. The adherent cell number was inhibited by 50 and 90% at 50 and 100 μg/ml doses of silymarin, respectively (Fig. 1E). The floaters displayed typical apoptotic morphology as indicated by cell condensation and fragmentation into apoptotic bodies. Such floaters did not re-attach or grow upon reseeding into silymarin-free fresh medium (data not shown).

HUVEC MMP-2 expression. Treatment with silymarin for 6 h, an exposure time that did not result in any visible morphological changes, led to a concentration-dependent decrease of MMP-2 (72 kD gelatinase A) in the conditioned media (i.e., secreted MMP) as detected by gelatin zymogram analyses (Fig. 2A). The extent of inhibition of the secreted MMP-2 by 100 μg/ml of silymarin was comparable to that induced by 25 μM curcumin, which has been shown to inhibit HUVEC MMP-2 and *in vitro* angiogenesis (26). In the cell lysate (Fig. 2B), 100 μg/ml silymarin inhibited MMP-2 by 67% and this effect was greater than that exerted by 25 μM curcumin, even though the secreted MMP-2 was decreased to the same extent by both compounds at the respective concentrations. At 50 μg/ml dose, silymarin did not decrease MMP-2 in the cell lysate even though it decreased the secreted MMP-2 by as much as 63%, indicating that at this level, silymarin might only inhibit the secretion of MMP-2 from the cells but not the cellular level. Incubation of the control medium (MMP-2 containing) with silymarin directly in the test tube did not inhibit its zymographic activity (data not shown), indicating a cellular dependent process for the inhibitory action on MMP-2 secretion and expression by silymarin.

In vitro angiogenesis on Matrigel by HUVEC. In experiments assessing the inhibitory effects on capillary tube formation, silymarin exposure, commenced at the time of seeding HUVEC onto Matrigel,

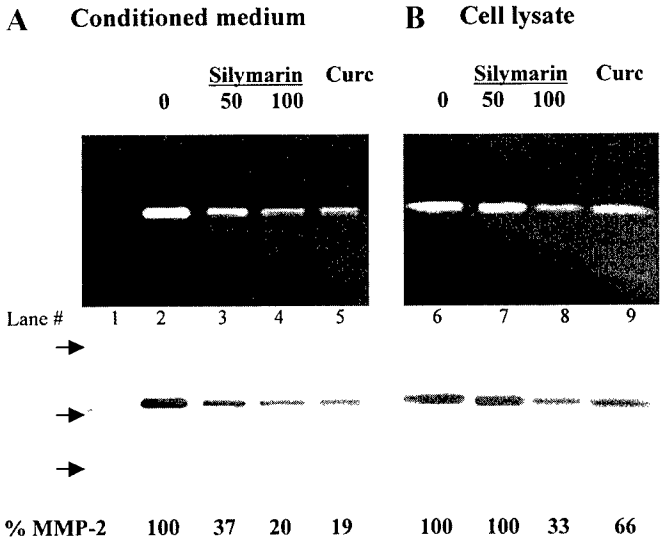

FIG. 2. Effect of silymarin or curcumin on secreted (A) and cell-associated (B) matrix metalloproteinase-2 detected by gelatin substrate gel zymography. HUVEC were treated in serum-free medium supplemented with 100 μg/ml of ECGS with silymarin or curcumin for 6 h. The conditioned media (A, lanes 2–5) and cell lysates (B) were analyzed on gelatin I impregnated substrate gels. Silymarin concentrations were 50 and 100 μg/ml. Curcumin treatment concentration was 25 μM. Inverted images of the zymograms (lower panels) were used for densitometric quantitation. The relative pixel density for the 72 kD gelatinase A/MMP-2 was shown below each lane. Arrowheads on the left mark position of molecular weight standards corresponding to (from top) 97, 66, and 47 kD. Lane 1 was serum-free medium as a blank control.

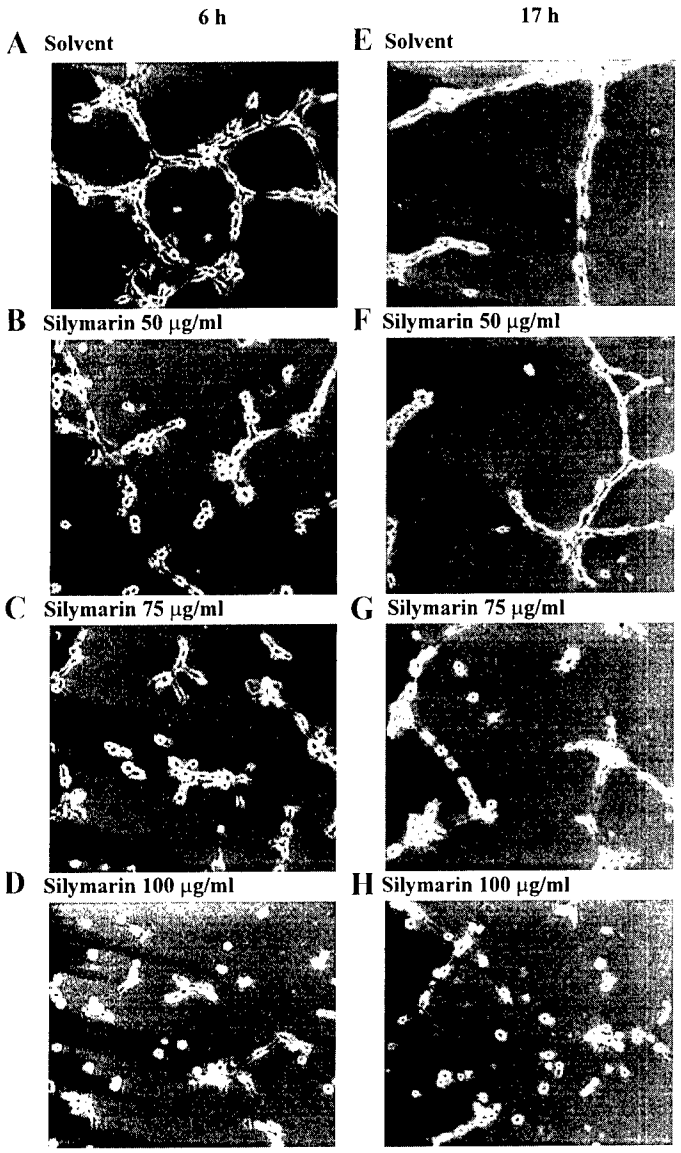

FIG. 3. Effect of silymarin on HUVEC capillary tube formation (*in vitro* differentiation) on Matrigel. HUVEC (20,000 cells/per well) in medium containing 10% serum was seeded into Matrigel pre-coated 24-well plate and treated with DMSO/ethanol solvent vehicle (A, E) or increasing concentrations of silymarin (B–D, F–H). Representative phase contrast photomicrographs (100× magnification) were taken at 6 h (A–D) and 17 h (E–H) after seeding. Each experiment condition was performed in duplicate wells and the experiments were repeated twice.

concentration-dependently inhibited tube formation at both 6 (Figs. 3A–D) and 17 h (Figs. 3E–H), achieving almost a complete block at the 100 μg/ml dose. Silymarin exposure of pre-formed tubes led to the retraction of cells and capillary disintegration (Fig. 4B versus 4A). The efficacy of silymarin at 100 μg/ml was comparable to that of 25 μM curcumin (Fig. 4C).

VEGF secretion by cancer epithelial cells. Silymarin treatment of DU145 human prostate carcinoma cells for 6 h decreased the secreted (in conditioned medium) VEGF content in a concentration dependent manner, resulting in a complete block by the 100 μg/ml dose (Table 1). Such inhibitory effect was observed in the absence of a reduction of the cell lysate VEGF content (Table 1). In human breast cancer cells, silymarin exposure reduced VEGF level in conditioned media in both MDA-MB 468 and MCF-7 cell lines (Table 1). The impact of silymarin on the cellular VEGF content was similar to that on DU145 cells, i.e., in MCF-7 cells as well as MDA-MB 468 cells at low to intermediate exposure levels, a reduction of secreted VEGF level was not associated with decreased cellular

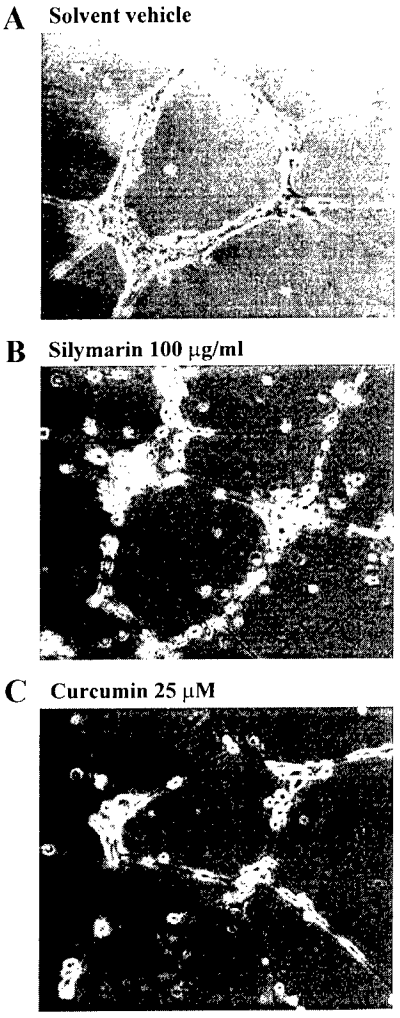

FIG. 4. Effect of silymarin or curcumin treatment on preformed HUVEC tubes. HUVEC (40,000 per well) were seeded into Matrigel pre-coated 24-well plate for 6 h for tube formation. The unattached cells and conditioned medium were removed and the tubes washed in fresh medium. The preformed tubes were treated with DMSO/ethanol vehicle (A), silymarin (B, 100 μg/ml) or curcumin (C, 25 μM). The cells were fixed in 1% glutaldehyde at 20 h after seeding and representative phase-contrast photomicrographs (100× magnification). Each experiment condition was performed in duplicate wells and the experiment was repeated twice.

TABLE 1

Effects of Silymarin Treatment on Vascular Endothelial Growth Factor (VEGF) Content in Conditioned Media (Secreted) and in Prostate and Breast Cancer Cell Lysates

Cell line	Silymarin μg/ml	Exposure time, h	VEGF in medium pg/flask	VEGF in lysate pg/flask
DU145	0	6	4272 ± 516[a1,2]	640 ± 54[c]
	25	6	4128 ± 480[a]	760 ± 16[b]
	50	6	2658 ± 264[b]	894 ± 10[a]
	100	6	72 ± 1[c]	758 ± 22[b]
MDA-MB-468	0	5	7815 ± 480[a]	1112 ± 24[a]
	25	5	6150 ± 165[b]	1222 ± 42[a]
	50	5	5300 ± 105[c]	1140 ± 26[a]
	100	5	2590 ± 65[d]	742 ± 14[b]
MCF-7	0	6	3420 ± 120[a]	455 ± 24
	50	6	3156 ± 246[a]	447 ± 9
	100	6	2178 ± 102[b]	501 ± 22

[1] Mean ± sd; $n = 3$ replicates.
[2] Data were analyzed by one-way ANOVA. Dissimilar superscripts indicate significant difference between means ($P < 0.05$).

VEGF content (Table 1). The exception was MDA-MB cells treated with 100 μg/ml silymarin where cellular VEGF content was decreased. In time course experiments, the secretion of VEGF was significantly decreased at 1 h of exposure to silymarin in both DU145 and MDA-MB 468 cells (Fig. 5). The inhibitory effects in all three cell lines were observed in the absence of morphological changes such as cell retraction, rounding, detachment or cytoplasmic vaccuolation.

DISCUSSION

A number of recent studies by Agarwal and associates (15–24) have shown that silymarin possesses significant chemopreventive and anti-cancer activity. Although cell culture studies have revealed many insights concerning the potential direct effects of silymarin exposure on cancer epithelial cells with respect to growth and survival signaling and cell cycle regulation, there has been no published work to address the potential impacts of silymarin on vascular endothelial cells and angiogenesis. The results of the present study support a potential anti-angiogenic activity of silymarin. Because tumor epithelial cells *in vivo* depend on angiogenesis to provide nutrients for their growth and survival, it is plausible that an anti-angiogenic effect may play a primary role in mediating the cancer chemopreventive activity of silymarin.

In the present study, first, silymarin inhibited endothelial cell growth and survival through induction of apoptosis in a concentration dependent manner (Fig. 1). Because angiogenic factor-stimulated proliferation of endothelial cells is crucial for capillary sprouting, growth inhibition and apoptosis induction can be one mechanism for silymarin to inhibit angiogenic response. Second, silymarin inhibited endothelial MMP-2 secretion and expression (Fig. 2) and such an effect occurred rapidly prior to the onset of any morphological changes. Because matrilytic activity of angiogenically-stimulated endothelial cells via MMP-2 is another important requirement for capillary sprouting (28–31), the inhibition of MMP-2 secretion and expression by silymarin may provide an inhibitory mechanism on angiogenesis independent of and/or in addition to endothelial growth arrest and apoptosis. Furthermore, silymarin inhibited *in vitro* capillary formation on Matrigel, a process requiring cell-matrix interaction, inter-cellular communications as well as cell motility, to name a few. It was noteworthy that the inhibitory effect on tube formation manifested whether the treatment was initiated simultaneous with seeding cells on the Matrigel (Fig. 3) or when the tubes had preformed (Fig. 4). These results support an anti-

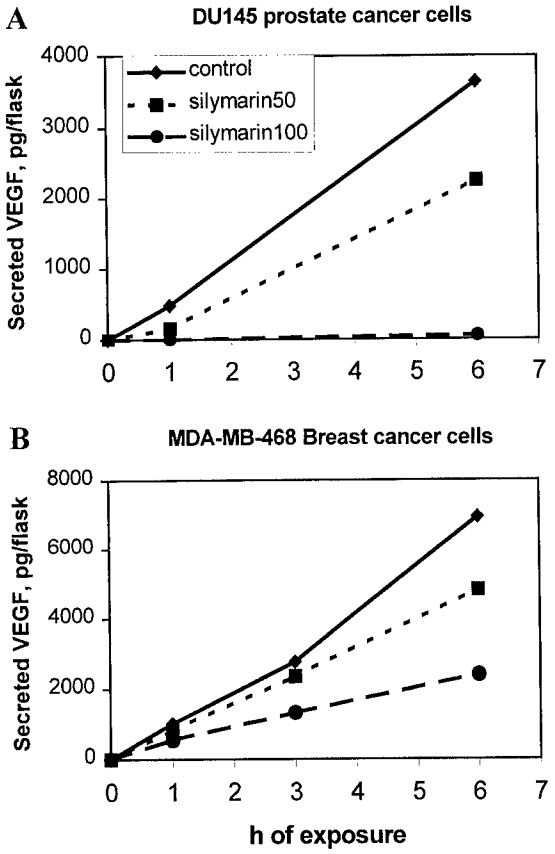

FIG. 5. Representative time course of silymarin effects on VEGF secretion by DU145 prostate cancer cells (A) and MDA-MB-468 breast cancer cells (B) in serum-free media. Confluent cells in T25 flasks were treated with solvent vehicle (DMSO/ethanol) or silymarin in 6 ml serum-free medium. At designated time points, 1-ml aliquots of conditioned media were taken for VEGF assay by ELISA. Each data point represents the mean of triplicate measurements. SD < 5% of respective means.

angiogenic potential for silymarin through multifaceted effects on endothelial proliferation and survival and matrix degradation activity and the capillary differentiation process. Work is in progress to further substantiate an anti-angiogenic effect of silymarin in other human endothelial cells in culture and in *in vivo* models.

In addition to these inhibitory effects on endothelial responses and *in vitro* angiogenesis, silymarin also exerted a rapid inhibitory action on the secretion of VEGF by cancer epithelial cells (Table 1 and Fig. 5). VEGF, also known as vascular permeability factor (VPF) (32, 33), plays several critical roles in vasculogenesis as well as angiogenesis. Its expression is so crucial that germ-line knockout of even one VEGF allele leads to embryonic lethality and homozygous knockout embryonic stem cells are incapable of forming tumors (34, 35). Whereas overexpression of VEGF is linked to increased angiogenesis and more aggressive tumor behavior (36, 37), anti-angiogenic interventions based on VEGF antibodies or interference of signal transduction through its receptors (38–42) have been shown to result in the inhibition of tumor growth and induction of endothelial apoptosis. Transformed epithelial cells have been shown to be the major source of VEGF expression in many types of solid cancers (43–46), however, recent data suggest that stromal cells and even vascular endothelial cells may also express VEGF in the hypoxic angiogenic microenvironment of tumors (47). These findings are supported by the observations that certain oncogenic mutations constitutively upregulate VEGF expression (48–51), and that cancer epithelial hypoxia, as a result of dysregulated cellular proliferation (5), is a potent *in vivo* inducer of VEGF expression (52, 53). The inhibitory effect of silymarin on secretion of VEGF in cancer epithelial cells, therefore, may be an important mechanism to negatively regulate the angiogenic switch of avascular lesions, further contributing to the overall control of lesion growth and progression.

The manners by which epithelial VEGF and endothelial MMP-2 were inhibited by silymarin are noteworthy and suggestive of a commonality with regard to the mechanisms of action by silymarin on these secretory proteins. In DU145 and MCF-7 cancer cell lines, silymarin exposure decreased secreted VEGF in the conditioned media without a reduction of cellular VEGF protein level (Table 1). In the MDA-MB468 cell line, exposure at low to intermediate levels of silymarin (25 or 50 μg/ml) decreased secreted VEGF level without lowering the cellular VEGF content, and only at the higher exposure level (100 μg/ml) a reduction of cellular VEGF level was observed (Table 1). This pattern was similarly to that observed for HUVEC MMP-2 expression in that an intermediate level of silymarin exposure (50 μg/ml) significantly decreased secreted MMP-2 level without a change in cellular MMP-2 (Fig. 2). These results from both epithelial and endothelial cells suggest that a primary action of silymarin may involve preferential targeting of the secretion and/or export (exocytosis) of these proteins critical for angiogenic switch regulation. We are currently investigating such mechanisms.

When the results of the present study showing cell death effect of silymarin on HUVEC were compared to those published by us showing anti-proliferative, but not cytotoxic and apoptotic effects, in several different human carcinoma and normal epithelial cells (20–24), it is important to emphasize here that apoptotic effect of silymarin is possibly specific to vascular endothelial cells. Based on these results, there is a possibility that on one hand silymarin is an anti-proliferative and a differentiation-inducing agent for cancer epithelial cells and on the other hand is both an anti-proliferative and an apoptogenic agent for vascular endothelial cells that are involved in neo-vascularization. These dual effects of silymarin possibly make it a useful agent for the prevention and therapy of epithelial cancers in humans.

In summary, this study, for the first time, documents the inhibitory actions of silymarin on several angiogenic responses, including growth and survival, MMP-2 expression and *in vitro* angiogenesis, of vascular endothelial cells as well as an inhibitory effect on the secretion of a primary angiogenic cytokine VEGF by cancer epithelial cells. The anti-angiogenic activity reported in this paper combined with the previously published multi-faceted broad spectrum anti-cancer effects of silymarin support the merit of further investigations to assess and define its cancer chemopreventive and/or therapeutic potential for humans.

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Appendix 5

ELSEVIER

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Cell Signaling and Regulators of Cell Cycle as Molecular Targets for Prostate Cancer Prevention by Dietary Agents

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ABSTRACT. Prostate cancer (PCA) is the most common invasive malignancy and leading cause (after lung) of cancer deaths in males. Since PCA is initially androgen-dependent, strategies are targeted toward androgen depletion for its control. However, tumor re-growth mostly occurs following this modality, and is androgen-independent. A loss of functional androgen receptor and an enhanced expression of growth factor receptors (e.g. erbB family members) and associated ligands have been shown to be the causal genetic events in PCA progression. These genetic alterations lead to an epigenetic mechanism where a feed-back autocrine loop between membrane receptor (e.g. epidermal growth factor receptor [erbB1] and associated ligand (e.g. transforming growth factor-α) results in an enhanced activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2) as an essential component of the uncontrolled growth of PCA at an advanced and androgen-independent stage. Together, we rationalized that inhibiting these epigenetic events would be useful in controlling advanced PCA growth. Dietary polyphenolic flavonoids and isoflavones are being studied extensively as cancer-preventive and interventive agents. Therefore, we focused our attention on silymarin, genistein, and epigallocatechin 3-gallate (EGCG), present in milk thistle, soy beans, and green tea, respectively. The effect of these agents was assessed on the erbB1-Shc-ERK1/2 signal transduction pathway, cell cycle regulatory molecules, and cell growth and death. In androgen-independent human prostate carcinoma DU145 cells, silymarin, genistein, and EGCG resulted in a significant to complete inhibition of transforming growth factor-α-caused activation of membrane receptor erbB1 followed by inhibition of downstream cytoplasmic signaling target Shc activation and a decrease in its binding with erbB1, without an alteration in their protein expression. Silymarin and genistein also inhibited ERK1/2 activation, suggesting that these agents impair the activation of erbB1–Shc–ERK1/2 signaling in DU145 cells. In the case of EGCG, a further increase in ERK1/2 activation was observed that was related to its pro-oxidant and apoptotic activities. Silymarin, genistein, and EGCG also resulted in a significant induction of Cip1/p21 and Kip1/p27 and a decrease in cyclin-dependent kinase (CDK) 4, but a moderate inhibition of CDK2, cyclin D1, and cyclin E was observed. An enhanced level of Cip1/p21 and Kip1/27 also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein, and EGCG also resulted in strong cell growth inhibition at lower doses, and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein also showed cell death. A more profound cytotoxic effect was observed in the case of EGCG, with strong cell death at lower doses and complete loss of viability at higher doses. Together, these results suggest that cell signaling and regulators of cell cycle are potential epigenetic molecular targets for prostate cancer prevention by dietary agents. More studies, therefore, are needed with these agents to explore their anticarcinogenic potential against human prostate cancer. BIOCHEM PHARMACOL 60;8:1051–1059, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cell signaling; cell cycle regulators, silymarin, epigallocatechin 3-gallate; genistein; growth factor receptor; erbB1, MAPK, Cip1/p21; Kip1/p27

PROSTATE CANCER

Prostate cancer (PCA)† is the most common non-skin malignancy in United States males, accounting for 41% of newly diagnosed cases, and is the second leading cause (after lung) of cancer deaths [1]. The prostate is one of the accessory sex glands in males. PCA, however, is a proliferation of only prostatic epithelial cells and is predominantly located in the peripheral zone of the prostate; it can also occur in the transition zone. Despite the high incidence of PCA, little is known with certitude about its etiology. The induction of PCA in humans has been viewed as a multistage process (as outlined in Fig. 1) involving progression from low histologic grade small latent carcinoma to higher-grade large metastasizing carcinoma. It is becoming clear that in the genesis of PCA a variety of pathogenetic pathways exists. Among the widely accepted risk factors for PCA are age, race ethnicity, and geographical dependence

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† *Abbreviations:* CDKs, cyclin-dependent kinases; CDKIs, cyclin-dependent kinase inhibitors; EGCG, epigallocatechin 3-gallate; EGFR (erbB1), epidermal growth factor receptor; ERK1/2, extracellular signal-regulated protein kinase 1/2; MAPK, mitogen-activated protein kinase; PCA, prostate cancer; RB, retinoblastoma; RTKs, receptor tyrosine kinases; and TGF-α, transforming growth factor-α.

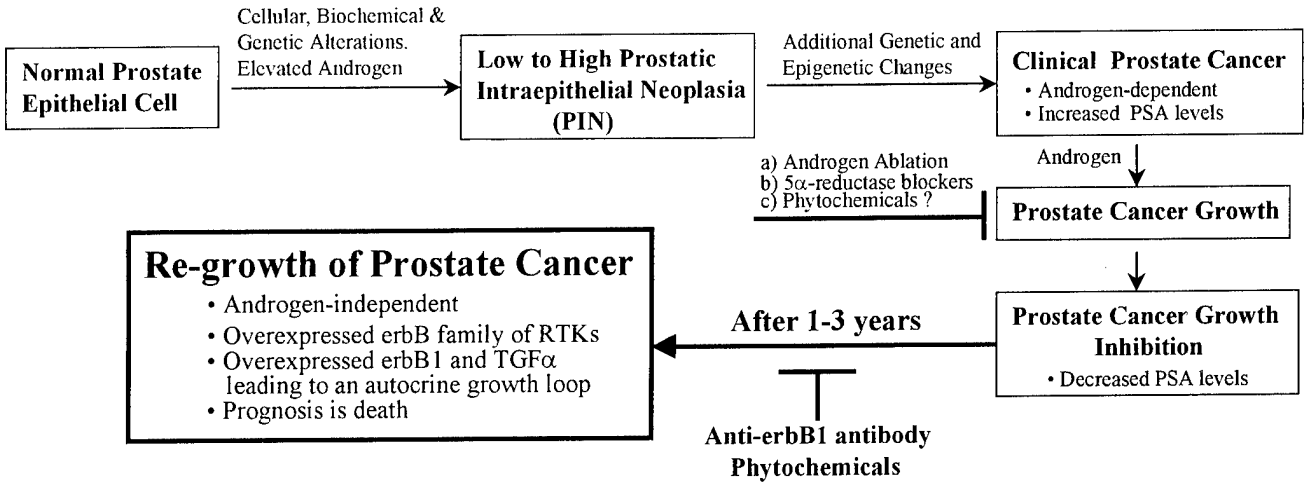

FIG. 1. Genesis of human prostate cancer is a multistep process. As shown in this figure, as an initial event, a normal epithelial prostate cell undergoes several cellular, biochemical, and genetic alterations leading to the formation of low- followed by high-grade prostatic intraepithelial neoplasia. The growth of transformed prostate epithelial cell is an elevated androgen-dependent phenomenon. Additional genetic and epigenetic changes in high-grade prostatic intraepithelial neoplasia lead to clinical prostate cancer that is androgen-dependent initially and can be monitored by an elevated serum prostate specific antigen (PSA) level. Since the malignancy is androgen-dependent at this stage, androgen deprivation and 5α-reductase blockers are extensively used to control and manage the disease at this point [5]. We have also reported that phytochemicals such as silymarin inhibit PSA levels regulated by both serum and androgen, causing strong inhibition of growth [43]. These approaches lead to the inhibition of PCA growth, which could be related to a decrease in serum PSA. Unfortunately, 1–3 years following these treatments, cancer re-growth mostly occurs that is totally androgen-independent and causally involves genetic alterations such as the overexpressed erbB family of RTKs and associated ligands. This situation leads to an epigenetic event of functional autocrine growth factor/receptor feed-back loop for the uncontrolled growth of malignant PCA cells as well as their metastasis to distant sites.

[2–4]. This malignancy is uncommon in the Asian population and high in the Scandinavian countries, with the highest incidence and mortality rates occurring in African American Males, the latter being twofold higher than in Caucasian American males [1–4]. Consistent with these reports, both epidemiology and laboratory studies have also suggested that diet and androgen alter PCA risk via a common etiologic pathway [2, 3]. It is becoming increasingly clear that androgens are involved in PCA pathogenesis and that cell division in the prostate is controlled by testosterone following intracellular conversion to its reduced form dihydrotestosterone [3] (Fig. 1).

The importance of testosterone in PCA could be further validated by studies showing that PCA rarely occurs in eunuchs or in men with a deficiency in 5α-reductase, the enzyme that converts testosterone to its active metabolite dihydrotestosterone [5]. Since cell division and proliferation in prostate is controlled by testosterone [5], inhibiting the biological effects of testosterone related to cellular proliferation in prostate tissue could be a novel approach for prevention against PCA. Indeed, androgen deprivation as well as 5α-reductase blockers have been extensively explored as a strategy for PCA prevention and therapy [5]. PCA patients treated with these therapies often experience remission of their PCA; however, tumor re-growth occurs which is largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation [6] (Fig. 1).

PROSTATE CANCER CHEMOPREVENTION

Since, even with the very high number of PCA diagnoses and related deaths in recent years, there are, other than surgery, no treatments for PCA in most patients, it is important that another strategy be developed. One approach is to prevent the occurrence of this disease in the first place. While many new classes of cancer chemopreventive agents are being evaluated in clinical trials for other malignancies, little success has been achieved in terms of PCA prevention. Chemoprevention of cancer is a means of cancer control where the occurrence of disease can be entirely prevented, slowed, or reversed by the administration of one or a combination of naturally occurring or synthetic compounds [7–12]. The overall goal of this modality is to reduce cancer incidence and multiplicity in the first place. The chemopreventive compounds are also known as anticarcinogens where the preventive approach includes intervention (or secondary prevention) of the conversion of precancerous lesions into malignant carcinomas [7]. Examples include prostatic intraepithelial neoplasia to prostate carcinomas, actinic keratosis to skin squamous cell carcinomas, etc. With regard to PCA prevention,

the preclinical efficacy of all-*trans*-*N*-(4-hydroxyphenyl-)retinamide, α-difluoromethylornithine, dehydroepiandrosterone, lirazole, lovastatin, oltipraz, finasteride (Proscar®), 9-*cis*-retinoic acid, etc., are being evaluated [13, 14]; all, however, are non-food-derived synthetic agents.

EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY OF RECEPTOR TYROSINE KINASES AND PROSTATE CANCER

The major goal of PCA research in recent years has been to elucidate and focus on the biology and molecular mechanisms of normal prostate and PCA to facilitate the design and conduct of molecular mechanism-based early phase prevention clinical trials. Indeed, several genetic alterations have been identified that lead to the induction and/or development of human PCA [13]. The early components of signal transduction pathways, specifically those of tyrosine kinases, were suggested to be of utmost significance for controlled cell growth and differentiation [15]. Ironically, a single genetic alteration in any of the cell signaling components results in continuous signaling, which in turn leads to uncontrolled cell growth (proliferation). RTKs participate in transmembrane signaling, whereas intracellular tyrosine kinases take part in signal transduction within the cell, including signaling to the nucleus [15]. Enhanced protein tyrosine kinase activity due to overexpression of RTKs and/or tyrosine kinase can lead to persistent stimulation by autocrinically secreted growth factors that in turn can lead to disease [15]. Enhanced activity of tyrosine kinases has been implicated in a wide variety of human malignancies; several studies have shown the increased expression of the EGFR or erbB family of RTKs in human malignancies, suggesting their role in the causation of such diseases [13, 15, 16]. With regard to human PCA, the aberrant expression of the erbB family of RTKs, such as EGFR (also known as erbB1), erbB2, and erbB3, has been demonstrated with strikingly high frequency in prostatic intraepithelial neoplasia and invasive PCA, both primary and metastatic [13, 17–19]. In addition, epidermal growth factor, TGF-α and erbB1 have been shown to be associated with the regulation of prostatic cell mitogenesis [20]. For example, hormone-independent prostate carcinoma cells commonly express high levels of erbB1 and TGF-α, thus making a functional autocrine feed-back loop for the hormone-independent growth of PCA [21, 22]. Employing the hormone-independent prostate carcinoma cell lines PC-3 and DU145, it has been shown that high-affinity, ligand-blocking monoclonal antibodies to erbB1 prevent its activation and also result in the growth inhibition of these cells [21–23]. Together, these studies imply that members of the erbB family of RTK-mediated signaling pathways may be contributory mechanisms for human PCA growth and metastasis [13, 16]; therefore, one practical and translational approach for the intervention of PCA could be to identify the inhibitors of the erbB family of RTK-mediated signaling pathway(s).

CELL CYCLE REGULATION

The significance of growth factors and the signaling pathway(s) initiated by them to regulate cell cycle progression in eukaryotes has been identified as an important component of their function [24–29]. Several studies have shown that cell signaling pathways determine cell growth as well as inhibition through cell cycle regulation [24–29]. However, cancer cells often display abnormalities in genes that govern the responses of these cells to external growth factors, growth factor receptors, proteins involved in the pathways of signal transduction in the cytoplasm, the nucleus, or both, and nuclear transcription factors [29]. In addition, defects in the regulation of cell cycle progression are thought to be one of the most common features of transformed cells [24]. Eukaryotic cell cycle progression is regulated by sequential activation and subsequent inactivation of a series of CDKs at different phases [30–32]. The activities of CDKs are positively regulated by cyclins and negatively by CDKIs [25]. A cyclin–CDK complex hyperphosphorylates RB, leading to its release from E2F [24, 27–29]. The free transcription factor E2F then activates the genes responsible for cellular proliferation by progression through G1 phase [24, 27–29]. Impairment of a growth-stimulatory signaling pathway (such as erbB1, raf, MAPK) has been shown to induce the expression of CDKIs such as Cip1/p21 and Kip1/p27 [23, 26, 27, 33]. An induced CDKI binds to and subsequently inhibits cyclin–CDK activity, which interferes with hyperphosphorylation of RB by keeping it in the hypophosphorylated form and bound to E2F, thereby blocking cell proliferation and inducing cell growth arrest [24, 28, 29].

WORKING HYPOTHESIS

Taken together, the above-summarized studies clearly suggest that progression of PCA depends on both genetic and epigenetic events where the multistep process leads from transformation of normal prostate epithelial cell over an androgen-dependent non-metastatic phenotype to a highly malignant metastatic androgen-independent malignancy (Fig. 1). At this stage, a functional autocrine growth factor/receptor loop plays a causal role in disease progression, and the prognosis is death of the host (Fig. 1). Whereas it is equally important to focus on both genetic and epigenetic events associated with this malignancy, one could argue that other than gene therapy, corrections in genetic defects may perhaps be more difficult to achieve. Accordingly, the most practical and translational approach to control human PCA could be to explore epigenetic events as potential molecular targets for control, prevention, and intervention of this deadly malignancy. In this regard, it is important to highlight here that epidemiological studies have shown that even with the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and associated mortality is low in Japan and other Asian countries [34]. These epidemiolog-

ical data further support the hypothesis that although the initiation of PCA is inevitable, targeting the associated epigenetic events could control its progression to clinical cancer. For example, it can be argued that despite the same incidence of latent prostatic carcinomas, the incidence of clinical PCA is low in Asian countries because of their dietary habits, among which is a regimen rich in several flavonoids and isoflavones that inhibits the progression of clinical PCA by modulating epigenetic events [35, 36]. It is important to emphasize here that several dietary agents including flavonoids and isoflavones have been implicated in diet-related protection against cancer [7–12] and that dietary habits in Asian countries largely include consumption of yellow–green vegetables, fruits, soybeans, green tea, etc. [11, 35, 36].

Taken together, the elements in the above discussion lead to the working hypothesis that phytochemicals present in the diet humans consume routinely as well as those in dietary supplements could be strong preventive and interventive agents against PCA by modulating epigenetic events associated with the progression of latent prostatic neoplasia to clinical malignancy. Accordingly, we structured a working model (shown in Fig. 2) to assess the effect of phytochemicals on these events in advanced and androgen-independent human prostate carcinoma DU145 cells. In constructing this study model, our rationale was that inhibition of erbB1 activation (which is constitutively active in DU145 cells and in advanced PCA for that matter) will lead to inhibition of downstream signaling targets such as Shc (in a classical erbB1-Shc-Grb2/SOS-ras-raf-ERK1/2 signaling cascade) that will ultimately inhibit ERK1/2-mediated mitogenic signaling followed by an increase in CDKI, causing an inhibition of CDK activity. These effects will impair the hyperphosphorylation of RB, keeping it in the hyperphosphorylated form and bound to transcription factor E2F, thus leading to cell growth arrest. Initially, we focused our efforts on assessing the effect of silymarin using the model system detailed in Fig. 2. These studies (the results are detailed later) showed that treatment of prostate carcinoma DU145 cells with silymarin at 100–200-μM doses inhibits erbB1-Shc-ERK1/2 mitogenic signaling and modulates cell cycle regulators, leading to a G1 arrest and inhibition of cell growth and colony formation. Based on these exciting data, we next asked the question whether these important findings could be extended to other flavonoids and isoflavones with cancer-preventive potential such as EGCG and genistein. A brief description for the selection of these agents is provided in the following paragraph.

Fruits, vegetables, and common beverages, as well as several herbs and plants with diversified pharmacological properties, have been shown to be rich sources of microchemicals with the potential to prevent human cancers [7–12]. Among these, naturally occurring flavonoids and isoflavones have received increasing attention in recent years [7–12]. Accordingly, the major ongoing research

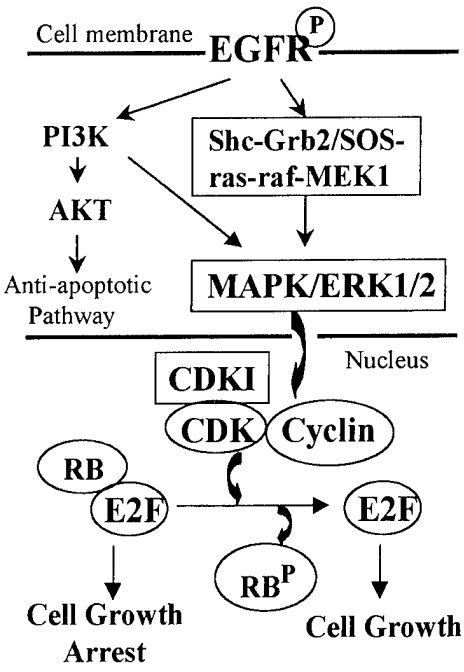

FIG. 2. A study model showing an interaction of mitogenic cell signaling with cell cycle regulators for cell growth. As shown in this figure, following EGFR activation (phosphorylation), several different mitogenic and cell survival pathways are activated in the cytoplasm. In a classical set-up, EGFR activation leads to recruitment of adoptor protein Shc followed by its activation and binding to Grb2/SOS that causes activation of ras-raf-MEK1-ERK1/2 mitogenic signaling [15, 62, 63]. Consistent with this signaling pathway and an autocrine growth factor/receptor feedback loop, MAPK/ERK1/2 is constitutively active in advanced and androgen-independent PCA, with this activation also causally associated with this malignancy at advanced stage [64, 65]. This and other mitogenic signaling cascades regulate cell cycle progression, possibly by activating CDKs that together with their catalytic subunit cyclins hyperphosphorylate RB, making transcription factor E2F free for cell growth [23–29, 33]. This model suggests that inhibition of erbB1 (EGFR) activation will lead to inhibition of the classical erbB1-Shc-Grb2/SOS-ras-raf-ERK1/2 signaling cascade that will ultimately inhibit ERK1/2-mediated mitogenic signaling, followed by an increase in CDKI and the resulting inhibition of CDK activity. These effects will impair the hyperphosphorylation of RB, keeping it in the hypophosphorylated form and bound to transcription factor E2F, thereby blocking cell proliferation and inducing cell growth arrest. Indeed, convincing evidence has been provided in recent studies from our laboratory and by other investigators that this is the case when phytochemicals and anti-EGFR antibody are used [23, 62, 66]. MEK1, MAPK kinase; P13K, phosphatidylinositol 3-kinase.

project in our laboratory has been to assess the effect of the cancer-preventive phytochemicals silymarin (flavanone), genistein (isoflavone), and EGCG (flavanol) (Fig. 3) on epigenetic events involved in uncontrolled growth of advanced and androgen-independent PCA. Silymarin is a naturally occurring agent present in milk thistle (*Silybum marianum*) and is used clinically in Europe, and more recently in Asia and the United States, for the treatment of liver disease [37]. It is also sold as a dietary supplement in

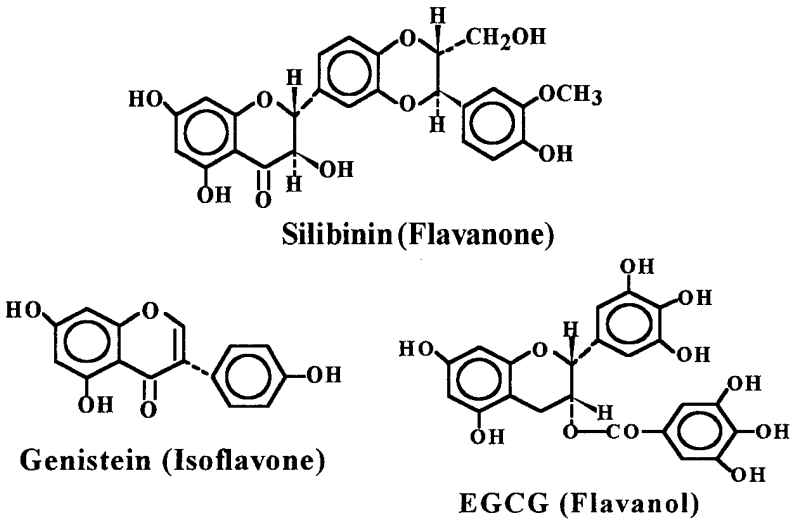

FIG. 3. Chemical structure of phytochemicals currently being studied for their efficacy on cell signaling and cell cycle regulators in human prostate carcinoma cells as summarized in the present overview.

the U.S.A. and Europe. Silymarin is non-toxic in acute, subchronic, and chronic tests in different animals and has no known LD_{50} [37]. In recent years, several studies from our laboratory have shown the cancer-preventive effects of silymarin in long-term tumorigenesis models and its anticarcinogenic activity in human prostate, breast, and cervical carcinoma cells [38–43]. Genistein is another dietary agent present in soybeans (*Glycine max*) [44] and has received much attention as a potential anticarcinogenic agent due to its effect on a number of cellular processes [45]. Several epidemiological and animal tumor studies have shown the preventive effects of genistein against various cancers [44, 46]. With regard to PCA, the anticarcinogenic and cancer-preventive effects of genistein have been extensively studied using cell and organ cultures and animal models; its efficacy is also being evaluated in PCA patients [47–55]. Tea (*Camellia sinensis*) is one of the most common beverages all over the world. Several studies from our group and by others have shown the cancer-preventive and anticarcinogenic effects of tea polyphenols on various cancers including skin, lung, esophagus, stomach, liver, intestine, pancreas, breast, and prostate [11, 12]. As a major component, EGCG constitutes ~50% (w/w) of the total green tea extract and has been implicated in both the cancer-preventive and anticarcinogenic effects of green tea [11, 12, 56–61].

EFFECT OF SILYMARIN ON EPIGENETIC EVENTS IN DU145 CELLS

For these studies, two different approaches were used. First, studies were done in serum-starved DU145 cells to assess the effect of ligand-caused activation of erbB1-Shc-MAPK/ERK1/2 mitogenic signaling, and secondly to determine the effect of silymarin on constitutive activation of the same signaling pathway and its association with cell cycle regulators. Whereas the first set of studies was useful in defining the mechanistic aspect of the study, the later studies represent the clinical PCA situation where these prostate carcinoma cells have autonomous growth advantage due to the ligand–receptor autocrine feed-back loop. In the first set of studies, treatment (with two medium changes at 12-hr intervals to remove ligand secreted via autocrine mechanism) of 36-hr serum-starved DU145 cells with silymarin resulted in a highly significant, dose-dependent inhibition of TGF-α-mediated activation of erbB1, but no change in its protein levels. Silymarin treatment of cells also resulted in a significant decrease in tyrosine phosphorylation of an immediate downstream target of erbB1, the adapter protein SHC, together with a decrease in its binding to erbB1. In other studies under similar treatment conditions, silymarin also showed a dose-dependent inhibition of TGF-α-caused activation of MAPK/ERK1/2 without any change in ERK1/2 protein levels. In additional studies, treatment of cells grown in 10% serum with different doses of silymarin also resulted in significant inhibition of constitutive tyrosine phosphorylation of both erbB1 and Shc followed by ERK1/2, but no change in their protein levels. Together, these results showed that silymarin impairs erbB1-Shc-ERK1/2-mediated mitogenic signaling in DU145 cells [62] (Fig. 4). In the erbB1-mediated mitogenic signaling pathway, via activation of Shc-Grb2-ras-raf, the ultimate cytoplasmic target is MAPK/ERK1/2, which following its activation translocates to the nucleus where it in turn activates transcription factors for cell growth and proliferation [15, 63]. In the case of advanced and androgen-independent PCA, several studies have shown genetic alterations resulting in an enhanced expression for erbB1 and associated ligand that leads to an epigenetic mechanism of autocrine growth loop via ligand/erbB1 interaction [20–23]. Together, these studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, recent studies have shown that MAPK/ERK1/2 is constitutively very active in DU145 cells and that epidermal growth factor, insulin-like growth factor-1, and protein kinase A activator significantly activate

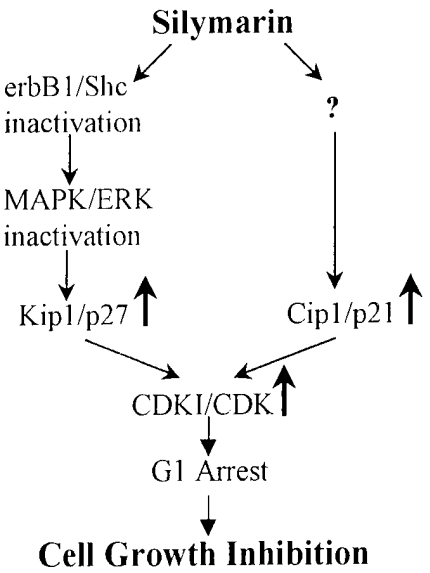

FIG. 4. A summary of the observed effects of silymarin on erbB-1-Shc-ERK1/2 signaling and cell cycle regulators in human prostate carcinoma DU145 cells. An inhibition of erbB1 activation by silymarin leads to a lack of Shc activation and binding to receptor followed by inhibition of ERK1/2 activation. This impairment of mitogenic signaling selectively induces Kip1/p27 followed by its increased binding to CDKs, causing an inhibition of their enzyme activity. This leads to an impairment of RB hyperphosphorylation, thereby keeping it hypophosphorylated and bound to E2F.* Together, these effects cause an arrest in cell cycle progression followed by cell growth inhibition.

MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells via the erbB1 receptor [64]. In addition, an increase in the activation of MAPK/ERK1/2 signaling was also reported more recently as human PCA progresses to a more advanced and androgen-independent malignancy [65]. Consistent with the involvement of activated MAPK/ERK1/2, possibly via the TGF-α/erbB1 autocrine loop, in the progression of advanced and androgen-independent human PCA, the results of our study showing that impairment of erbB1-Shc activation results in the inhibition of MAPK/ERK1/2 activation in DU145 cells by silymarin could be of great significance in further evaluating the effect of this dietary supplement in human PCA prevention and/or intervention.

In the studies analyzing the effect of silymarin on cell cycle regulatory molecules, silymarin treatment of cells grown in 10% serum resulted in a significant induction of the CDKIs Cip1/p21 and Kip1/p27, concomitant with a significant decrease in CDK4 expression but no change in the levels of CDK2, CDK6, and associated cyclin E and cyclin D1. Cells treated with silymarin also showed an increased binding of CDKIs with CDKs, together with a marked decrease in the kinase activity of CDKs and associated cyclins. In additional studies, treatment of cells grown in 10% serum with anti-EGFR monoclonal antibody clone 225 also resulted in a significant inhibition of constitutive tyrosine phosphorylation of both erbB1 and Shc, but no change in their protein levels. Furthermore, whereas silymarin treatment resulted in a significant increase in the protein levels of both Cip1/p21 and Kip1/p27, monoclonal antibody 225 showed an increase only in Kip1/p27 [62]. These findings suggest that the observed effect of silymarin on an increase in CDKI protein levels is mediated via inhibition of erbB1 activation only in the case of Kip1/p27; however, additional pathways independent of inhibition of erbB1 activation are possibly responsible for the silymarin-caused increase in Cip1/p21 in DU145 cells [62] (Fig. 4). In other studies, silymarin treatment also induced a G1 arrest in the cell cycle progression of DU145 cells, and resulted in a highly significant to complete inhibition of both anchorage-dependent and -independent growth of DU145 cells in a dose- and time-dependent manner. As summarized in Fig. 4, together these results suggest that silymarin may exert a strong anticarcinogenic effect against PCA and that this effect is likely to involve impairment of the erbB1-Shc-ERK1/2-mediated mitogenic signaling pathway, leading to an induction of CDKIs that inhibit the growth-promoting activity of CDKs, causing G1 arrest followed by cell growth inhibition [62].

EFFECTS OF GENISTEIN AND EGCG ON EPIGENETIC EVENTS IN DU145 CELLS: A COMPARISON WITH SILYMARIN

Based on the above findings with silymarin, we next wondered whether these important findings could be extended to other flavonoids and isoflavones with cancer-preventive potential such as EGCG and genistein. For these studies, 36-hr serum-starved DU145 cells were treated with similar doses (100–200 μM) of silymarin, genistein, and EGCG for 2 hr followed by ligand TGF-α for 15 min, cell lysates were prepared, and levels of activated signaling molecules (erbB1-Shc-ERK1/2) were analyzed by immunoprecipitation and immunoblotting. As summarized in Table 1 [66], treatment of cells with silymarin, genistein, and EGCG at 100–200 μM resulted in a complete inhibition of TGF-α-caused activation of erbB1, followed by a moderate to strong inhibition of Shc activation as well as its binding to erbB1 without an alteration in their protein levels. Silymarin and genistein also inhibited ERK1/2 activation in a dose-dependent manner without an alteration in protein levels. These results suggested that these phytochemicals impair erbB1-Shc-ERK1/2 signaling in DU145 cells [66]. In the case of EGCG, it was interesting to observe that it inhibited erbB1-Shc activation but further stimulated ERK1/2 activation. At the same time as the detailed mechanism of this effect is currently under investigation in our laboratory, initial observations suggest that this effect of EGCG could be related to its pro-oxidant and apoptotic activities [67].

In other studies, we assessed the effect of these three

* Tyagi A, Agarwal C and Agarwal R, unpublished observation.

TABLE 1. Summary of the effects of the phytochemicals silymarin, genistein, and EGCG on cell signaling and cell cycle regulators, leading to biological responses in human prostate carcinoma DU145 cells [adopted from Refs. 62 and 66]

Biological Effect	Silymarin	Genistein	EGCG
ErbB1 activation	Inhibited	Inhibited	Inhibited
Shc activation	Inhibited	Inhibited	Inhibited
Shc binding to erbB1	Inhibited	Inhibited	Inhibited
MAPK/ERK1/2 activation	Inhibited	Inhibited	Induced
Cip1/p21 protein levels	Induced	Induced	Induced
Kip1/p27 protein levels	Induced	Induced	Induced
CDK2	Moderate inhibition	No effect	No effect
CDK4	Inhibited	Inhibited	Inhibited
CDK6	No effect	Not done	Not done
Cyclin D1	Weak inhibition	Weak inhibition	No effect
Cyclin E	Weak inhibition	Weak inhibition	No effect
CDKI/CDK binding	Induced	Induced	Induced
CDK and cyclin kinase activities	Inhibited	Not done	Not done
Cell cycle progression	G1 arrest	Not done	G1 arrest
Cell Growth	Inhibited	Inhibited	Inhibited
Apoptosis	Moderately induced	Induced	Induced
Soft agar colony formation	Inhibited	Not done	Not done
Nude mice xenograft growth	Inhibited	Not done	Not done

phytochemicals on cell cycle regulators and the growth and death of DU145 cells. In these studies, as summarized in Table 1 [66], silymarin, genistein, and EGCG resulted in a significant induction of Cip1/p21 and Kip1/p27 and a strong decrease in CDK4, but only a moderate effect on CDK2 and cyclins D1 and E. An enhanced level of CDKIs also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein, and EGCG also resulted in 50–80% cell growth inhibition at lower doses and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein showed a cytotoxic effect causing 30–40% cell death. A more profound cytotoxic effect was observed in the case of EGCG, with 50% cell death at lower doses and a complete loss of viability at higher doses (Table 1).

CONCLUSION AND FUTURE GOALS

Concluding the overview provided in this communication, the convincing laboratory research findings summarized herein show an inhibitory effect of phytochemicals on erbB1-Shc-ERK1/2-mediated mitogenic signaling and on modulation of cell cycle regulators (specifically CDKIs, CDKs, cyclins, CDK activity) towards cell growth arrest and death. These studies form the basis for the question: what is the biological significance of the observed effect of these agents in terms of: a) human prostate carcinoma xenograft growth in nude mice; b) prostate cancer prevention and/or intervention in animals models; and c) human prostate cancer prevention/intervention in clinical trials? Currently, such studies are in progress in our program on "Phytochemicals and Cancer Prevention." However, based on studies already completed, it could be suggested that flavonoids and isoflavones are a class of dietary agents which should be studied in more detail to be developed as preventive and/or anticarcinogenic agents against human prostate cancer.

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Appendix 6

Impairment of erbB1 receptor and fluid-phase endocytosis and associated mitogenic signaling by inositol hexaphosphate in human prostate carcinoma DU145 cells

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Recently, we observed that epidermal growth factor receptor (EGFR or erbB1) endocytosis and associated mitogenic signaling occur in human prostate cancer (PCA) cells, suggesting that erbB1 endocytosis might be involved in advanced and androgen-independent PCA growth. Based on these findings, and the fact that aberrant expression of erbB family members is common in human prostatic intraepithelial neoplasia and invasive PCA, we reasoned that impairment of erbB1 endocytosis and associated mitogenic signaling might inhibit PCA growth. Inositol hexaphosphate (IP6) interacts with plasma membrane clathrin-associated protein complex 2 (AP2) and inhibits phosphatidylinositol 3-kinase (PI3K). As these are essential components of receptor-mediated and fluid-phase endocytosis, respectively, we reasoned that IP6 might impair erbB1 endocytosis and associated signaling in human PCA cells, leading to their growth inhibition. IP6 strongly to completely inhibited (26–100%; $P < 0.05$) transforming growth factor α-induced binding of activated erbB1 to AP2 in human PCA DU145 cells, demonstrating the impairment of the initial step in ligand-induced erbB1 endocytosis. IP6 treatment of cells resulted in a dose-dependent increase (1.8- to 7.7-fold compared with cells treated with ligand alone; $P < 0.05$) in levels of activated erbB1. These two findings suggest that the inhibitory effect of IP6 on receptor endocytosis is independent of its lack of effect on ligand-induced erbB1 activation. These effects of IP6, however, were associated with strong inhibition of ligand-induced Shc phosphorylation (77–84% decrease; $P < 0.05$) and its binding to erbB1 (58–100% decrease; $P < 0.05$). IP6 also significantly and dose-dependently inhibited fluid-phase endocytosis (19–52%; $P < 0.05$). It inhibited PI3K–AKT signaling pathway as an upstream response in its effect on the inhibition of fluid-phase endocytosis. The inhibition of erbB1 receptor and fluid-phase endocytosis, and associated signaling by IP6, was corroborated by very strong to complete inhibition (70–100%; $P < 0.05$) of extracellular signal-regulated protein kinase 1/2 activation by IP6. IP6 significantly ($P < 0.05$) inhibited anchorage-dependent and -independent inhibition (50–100% and 30–75%, respectively) in DU145 cells. Targeting the impairment of erbB1 endocytosis and associated mitogenic signaling by IP6 in advanced and androgen-independent human PCA DU145 cells could be a useful approach for treating PCA.

Abbreviations: AP2, plasma membrane clathrin-associated protein complex 2; EGFR (erbB1), epidermal growth factor receptor; ERK, extracellular signal-regulated protein kinase; HRP, horseradish peroxidase; IGF-1, insulin like growth factor 1; IP6, inositol hexaphosphate; MAPK, mitogen activated protein kinase; NDF, neu differentiation factor; PCA, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PIN, prostatic intraepithelial neoplasia; TGFα, transforming growth factor α.

Introduction

Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy, and second leading cause of cancer deaths in males in the USA (1,2). Induction of PCA is viewed as a multistage process, involving progression from small, latent carcinomas of low histological grade, to large, metastatic carcinomas of higher grade (2–5). The widely accepted risk factors for PCA are age, race, ethnicity, dietary habits, and androgen secretion and metabolism (2–5). Epidemiological data have revealed that environmental and behavioral factors are more important than genetic factors in determining overall cancer frequency among populations (reviewed in ref. 6). Consistent with these reports, diet and androgen have been shown to play a major role in the pathogenesis and promotion of PCA (2–5). The role of androgen in PCA is further supported by the fact that this malignancy rarely occurs in eunuchs or men with a deficiency in 5α-reductase, the enzyme that irreversibly converts testosterone to its active metabolite, dihydrotestosterone (7–9). Since the growth and development of PCA is initially androgen dependent, androgen deprivation has been extensively explored as a strategy for PCA prevention and therapy (7). PCA patients treated with androgen deprivation therapy often have remission of the disease, but tumor regrowth occurs, largely due to progression of initially androgen-dependent PCA cells to tumor cells that do not depend on androgen for their proliferation (8).

In addition to the loss of androgen dependence due to lack of androgen receptor and/or its function (10), interactions between functional autocrine or paracrine growth factors and growth factor receptors are major contributors to the multifactorial mechanisms of androgen independence in PCA (11–13). For example, advanced and metastatic human PCA cells express high levels of epidermal growth factor receptor (EGFR or erbB1) and transforming growth factor α (TGFα) (11–13). Furthermore, expression of erbB family members (e.g. erbB1, erbB2 and erbB3) is often abnormal in prostatic intraepithelial neoplasia (PIN) and in invasive PCA, both primary and metastatic (14–19). In fact, erbB family receptors are one of the few potential surrogate endpoint genetic markers that have been used for the screening of interventive agents against PCA in short-term phase II clinical trials (14–19). They have also been extensively explored as major potential molecular targets for PCA intervention, specifically in androgen-independent PCA (reviewed in ref. 20).

Activation of erbB1 by its ligand includes receptor dimerization, activation of intrinsic receptor tyrosine kinase activity, autophosphorylation of the receptor at the carboxyl terminus

and tyrosine phosphorylation of and/or association with intracellular signaling molecules such as Shc, and phosphatidylinositol 3-kinase (PI3K) (19–22). The binding of ligand to the receptor results in rapid disappearance of receptors from the cell surface. Receptor down-regulation is due to ligand-accelerated endocytosis and degradation of erbB1 (23,24). Morphological studies suggest that ligand increases receptor endocytosis by promoting receptor clustering into clathrin-coated pits on the plasma membrane and that this is followed by receptor internalization into clathrin-coated vesicles (25).

The ligand-dependent acceleration of receptor internalization is the rate-limiting step in receptor down-regulation and activation (26,27). The internalization process of receptor occurs through receptor-mediated endocytosis, where plasma membrane-coated pits function as sorting organelles selectively recruiting receptors that contain internalization sequences or 'codes' within their cytoplasmic domains (26,27). A major structural component of coated pits is the clathrin lattice anchored to cytoplasmic surface of the membrane by associated protein complexes or adaptors [plasma membrane clathrin-associated protein complex 2 (AP2)] (28). AP2 is the most widespread of the associated proteins found in coated vesicles derived from the plasma membrane (29), and has been shown to interact specifically with erbB family members (22,30). In addition to receptor-mediated endocytosis involving initial binding of activated receptor with AP2 for receptor internalization, fluid-phase endocytosis can occur, mediated via the PI3K–AKT–Rab5 pathway (31–35).

Taken together, the literature studies summarized above suggest that erbB1 endocytosis, and associated mitogenic and cell survival signaling, could be major events in the growth and proliferation of human prostate carcinoma cells. Indeed, in a recent study, we observed that both erbB1 receptor-mediated and fluid-phase endocytosis, and associated mitogenic and cell survival signaling, occur in human prostate carcinoma cells in the order of DU145 > PC3 > LNCaP (36). Based on these results, we reasoned that agents that impair receptor endocytosis and associated mitogenic/cell survival signaling could be useful for the treating human PCA.

Traditional Asian diets and those of vegetarians are not only high in starch and fiber, but are also rich in many bioactive compounds which are receiving increasing attention for the prevention and intervention of a wide variety of human cancers (20,37–43). Epidemiological data suggest that consumption of a high-fiber diet is associated with a reduction in breast, colon and prostate cancers (reviewed in ref. 44). The only types of high-fiber diet that have been consistently associated with a reduction in colon and breast cancers are cereals and legumes, which contain high levels of inositol hexaphosphate (IP6) (44). IP6 (also known as phytic acid) constitutes 0.4–6.4% (w/w) of most cereals, nuts, legumes, oil seeds and soybean; it is also a component of mammalian cells occurring at concentrations of 10–100 μM in both resting and stimulated cells (44,45). Several studies in recent years have shown the chemopreventive and anti-carcinogenic effects of IP6 against different cancers of epithelial and mesenchymal cell origin in both *in vivo* and *in vitro* models (reviewed in refs 44,46–49). It has been shown to be protective against colon (44,46,47), mammary (50,51), liver (52), lung (53) and skin (44) tumorigenesis, to inhibit the growth of mouse fibrosarcoma FSA-1 cell tumor xenograft in nude mice and to reduce the number of metastatic lung colonies and improve host survival (54). With regard to PCA, it has been shown that IP6 inhibits the growth and induces differentiation of human prostate carcinoma PC3 cells (55).

Mechanistic studies have shown that IP6 is involved in various signal transduction pathways. For example, it binds to clathrin assembly protein AP2 (56) and directly inhibits PI3K activation and activity (57); these are essential components in receptor-mediated and fluid-phase endocytosis, respectively. Based on the studies above described, we reasoned that IP6 would impair both AP2- and PI3K-mediated endocytosis, and thereby, associated cellular mitogenic responses in human PCA cells, and that this might be a novel approach for treating PCA.

Endocytosis is a carefully orchestrated process required for nutrition, down-regulation of surface receptors and maintenance of homeostasis (58). Some endocytosis proteins have been reported in human cancers, and enhanced endocytosis is associated with neoplastic transformation (59). Accordingly, we focused our efforts in this study to assess the impairment of erbB1 endocytosis and associated signaling by IP6 in human prostate carcinoma DU145 cells. Advanced and metastatic human PCA cells such as DU145 and PC3 lack functional androgen receptor and express high levels of erbB1 together with TGFα, leading to autonomous growth of cancer cells via an autocrine feedback loop (11–13,20). These cells, therefore, represent a valuable system to investigate erbB1 endocytosis and associated signaling, and in testing its impairment by potential preventive agents.

We show that IP6 inhibits (i) the binding of ligand-activated erbB1 to AP2, which may impair the erbB1–Shc–mitogen-activated protein kinase (MAPK) signaling pathway and (ii) ligand-induced activation of the PI3K–AKT signaling pathway. These effects of IP6 also resulted in the inhibition of prostate carcinoma DU145 cell growth.

Materials and methods

Materials

The human prostate carcinoma DU145 cell line was obtained from the American Type Culture Collection (Manassas, VA). IP6, horseradish peroxidase (HRP) and wortmannin were from Sigma–Aldrich Chemical Co. (St. Louis, MO). Human TGFα and all other cell culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Anti-erbB1 (EGFR), anti-Shc, anti-PI3K, anti-AKT, anti-phospho-AKT and anti-phosphotyrosine antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-AP2 antibody was from Affinity Bioreagents Inc. (Denver, CO). Anti-phospho-MAPK/ERK1/2 and anti-MAPK/ERK1/2 antibodies were from New England Biolabs (Boston, MA). Secondary antibodies (rabbit antibodies against mouse immunoglobulin and goat antibodies against HRP-conjugated rabbit immunoglobulins) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The enhanced chemiluminescence (ECL) detection system was from Amersham Corp. (Arlington Heights, IL).

Cell culture and treatments

Cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin–streptomycin under standard culture conditions in 100 mm dishes until they reached 70% confluency. Cells were then starved in serum-free medium for 36 h; during the last 2 h of starvation, they were treated with double-distilled water alone or with varying concentrations (0.25–2 mM) of IP6 dissolved in water or with 200 ng/ml wortmannin. The IP6 concentrations used in the present study were identical to those used in other studies (44,55,57). At the end of these treatments, cultures were treated with phosphate-buffered saline (PBS) or ligand TGFα (100 ng/ml medium) in PBS and incubated for 10 min at 37°C. Thereafter, medium was aspirated, attached cells were washed twice with cold PBS and cell lysates were prepared as detailed recently (20).

Immunoprecipitation and immunoblotting

For immunoprecipitation, cell lysates (200–500 μg protein) were clarified by protein A/G agarose for 1 h and then incubated with primary antibody directed against erbB1, Shc, PI3K or AKT for 4 h followed by addition of protein A/G agarose and overnight incubation at 4°C with rocking. Immunocomplexes

were washed three times with lysis buffer (20). For immunoblotting, immunocomplexes or cell lysates (20–80 μg protein) were denatured in sample buffer (2× buffer is 125 mM Tris pH 6.8, containing 4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% β-mercaptoethanol and 0.04% bromophenol blue), proteins were separated on SDS–polyacrylamide gels (8% or 12% gel) and transferred on to nitrocellulose membranes. The membranes were blocked with blocking buffer (10 mM Tris pH 7.5, containing 100 mM NaCl, 0.1% Tween 20 and 5% non-fat milk powder) at room temperature for 1 h, then incubated overnight with the appropriate primary antibody (at 1–2 μg/2 ml dilutions) directed against AP2, phosphotyrosine, erbB1, Shc, PI3K, AKT, phospho-AKT, MAPK or phospho-MAPK followed by the appropriate secondary antibody, and developed using an ECL kit (20). Autoradiograms of the western immunoblots were scanned using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using Scanimage Program (National Institutes of Health, Bethesda, MD). In each case, only representative blots are shown with reproducible findings in three or four independent experiments. The densitometric data shown in each case are mean ± SD of three or four independent experiments.

Fluid-phase endocytosis assay

As a measure of fluid-phase endocytosis, HRP uptake was assayed as described by Li and Stahl (60). Briefly, cultures in 35 mm dishes at 70–80% confluency were washed three times with serum-free α-MEM, and treated with varying concentrations of IP6 or 200 ng/ml wortmannin in α-MEM for 2 h at 37°C. HRP endocytosis was initiated by addition of 2 mg/ml HRP and 1% (w/v) bovine serum albumin at 37°C for another 1 h. To estimate HRP uptake, the cells were washed three times with PBS, trypsinized on ice for 20 min, washed again twice with PBS and lysed in 500 μl of lysis buffer (60). Cell lysates were assayed for HRP activity (60) and protein concentration was determined using the Bio-Rad DC protein assay according to the manufacturer's instructions. This experiment was repeated twice more, each done in duplicate.

Cell growth and soft agar colony formation assays

For cell growth, DU145 cells were plated at a density of 0.5×10^5 cells per 60 mm plate. After 24 h (denoted as day 0 of treatments), cells were fed with fresh medium and left untreated, or treated with IP6 dissolved at 0.25, 0.5, 1 or 2 mM (final concentration) in double-distilled water. The cultures were fed with fresh medium with or without the same concentrations of IP6 every other day up to the end of the experiment. Each treatment and time point had four plates. After various treatment times, cells were trypsinized and counted as described (20). Cell viability was assessed using the Trypan Blue dye exclusion method. This experiment was repeated twice more. The representative data shown in this report were reproducible in three independent experiments.

For soft agar colony formation, DU145 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin–streptomycin as detailed above. Soft agar colony formation assay was performed using six-well plates as described (20). Briefly, each well contained 2 ml of 0.5% agar in medium as the bottom layer, 1 ml of 0.38% agar in medium with ~1000 cells as the feeder layer, and 1 ml of 0.38% agar in medium with different concentrations of IP6 as the top layer. Three wells were used for each treatment. Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. The number of colonies was determined by counting them under an inverted phase-contrast microscope at ×100 magnification; a colony was counted as ≥16 cells. This experiment was repeated once more. The representative data shown were reproducible in two independent experiments.

Statistical analysis

The data were analyzed using one-way analysis of variance (ANOVA) performed with SAS/Proc GLM (SAS Institute, Cary, NC). If the ANOVA indicated at least one significant difference (using a *P*-value cut-off of 0.05), pairwise comparisons were performed. The within-experiment type I error rate was controlled at 0.05 using the Bonferroni correction for multiple comparisons. For three comparisons, if a difference is to be regarded as statistically significant, the Bonferroni correction requires a *P*-value of <0.0167 for any given pairwise comparison. Pairs of treatment groups referred to as statistically significant in the Results section are based on these *post hoc* tests.

Results

IP6 inhibits the binding of ligand-activated erbB1 to AP2 in DU145 cells

Using DU145 cells, we first explored the effect of IP6 on receptor endocytosis process by analyzing the binding of ligand-activated erbB1 to AP2. As shown in Figure 1A (lane 1), immunoprecipitation with anti-erbB1 antibody and immunodetection with anti-AP2 antibody showed that 36 h of serum starvation of DU145 cells resulted in diminished binding of erbB1 to AP2. This observation suggested that erbB1–AP2 binding is an activated erbB1-associated phenomenon that is reduced to very low levels after 36 h serum starvation. After starved cells had been treated with TGFα (100 ng/ml) for 10 min, however, they showed very strong erbB1–AP2 binding (Figure 1A, lane 2). Pretreatment of cultures with 0.25, 0.5 or 1 mM IP6 during the last 2 h of starvation followed by treatment with TGFα at the same concentration and for the same time resulted in a strong decrease in the binding of erbB1 to AP2 (Figure 1A, lanes 3–5). Densitometric analysis of the blots from four independent experiments (Figure 1B) showed that, compared with ligand alone, pretreatment with 0.25, 0.5 or 1 mM IP6, followed by ligand activation, resulted in a statistically significant ($P < 0.05$) decrease (26%, 50% and 100%, respectively) in the binding of erbB1 to AP2 (Figure 1B). Conversely, treatment of serum-starved cultures with a PI3K inhibitor, wortmannin, at 200 ng/ml followed by TGFα did not show any change in erbB1 binding to AP2

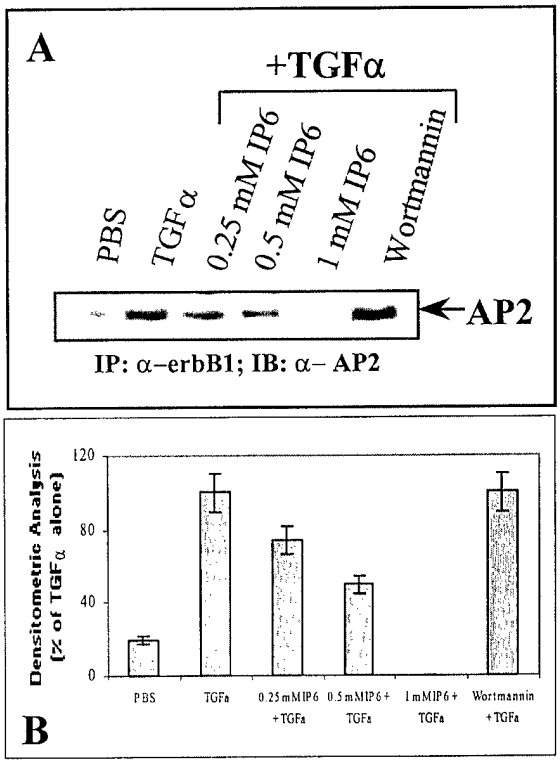

Fig. 1. IP6 impairs erbB1 receptor-mediated endocytosis by inhibiting the binding of AP2 to erbB1 in DU145 cells. Cells at 70–80% confluence were starved of serum for 36 h; during the last 2 h of starvation they were treated with double-distilled water, or 0.25, 0.5 or 1 mM IP6 dissolved in water, or 200 ng/ml wortmannin. At the end of these treatments, cells were treated with PBS or TGFα (100 ng/ml) in PBS for 10 min at 37°C, and cell lysates were prepared as detailed in Materials and methods. ErbB1 was immunoprecipitated using an anti-erbB1 antibody, and then immunoprecipitates were subjected to SDS–PAGE followed by western blotting. (**A**) Membrane was probed with an anti-AP2 antibody followed by peroxidase-conjugated appropriate secondary antibody, and visualized by the ECL detection system. The treatment in each lane is as marked in the figure. One representative blot is shown from four independent studies with reproducible findings. (**B**) Densitometric analysis data (mean ± SD) from four independent experiments.

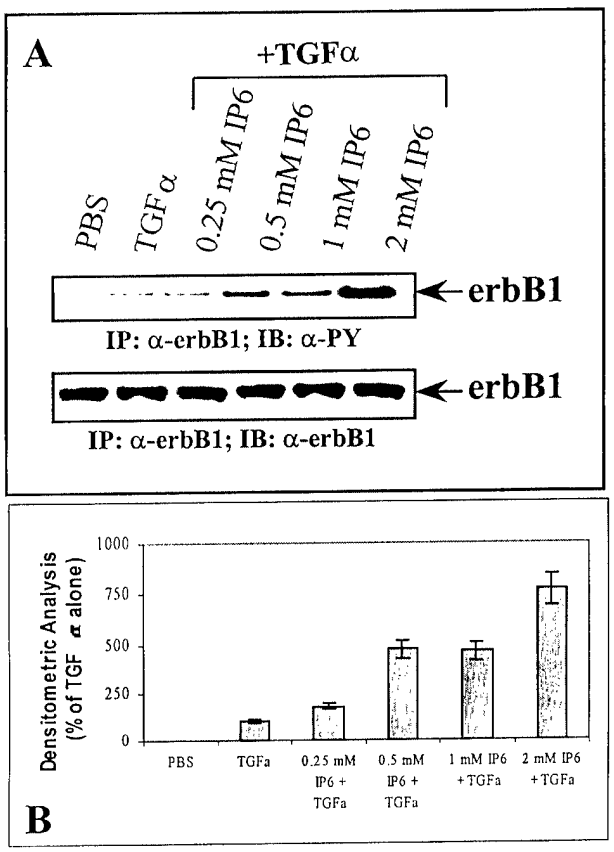

Fig. 2. Impairment of erbB1 receptor-mediated endocytosis by IP6 does not affect erbB1 activation in DU145 cells. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. ErbB1 was immunoprecipitated using anti-erbB1 antibody and immunoprecipitates were then subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blotting. (**A**) Membrane was probed with anti-phosphotyrosine (upper panel) or anti-EGFR (lower panel) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualized using the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (**B**) Densitometric analysis data (mean ± SD) from three independent experiments.

(Figure 1A, lane 6), suggesting that, after ligand treatment, the erbB1 receptor–AP2 interaction is PI3K independent.

Inhibition by IP6 of ligand-activated erbB1–AP2 binding is independent of erbB1 activation in DU145 cells

We next assessed the involvement of erbB1 activation in this ligand-induced binding of erbB1 to AP2. As shown in Figure 2A (top panel), serum starvation of cells for 36 h completely inactivated erbB1 (lane 1). However, treatment of starved cultures with TGFα for 10 min resulted in a marked activation of erbB1 receptor (Figure 2A, lane 2). Pretreatment of cultures with 0.25, 0.5, 1 or 2 mM IP6 for 2 h followed by ligand treatment under identical conditions resulted in a strong increase in erbB1 tyrosine phosphorylation (Figure 2A, lanes 3–6). Densitometric analysis of the blots from three independent experiments (Figure 2B) showed that, compared with ligand alone, pretreatment with IP6 at 0.25, 0.5, 1 or 2 mM followed by ligand stimulation resulted in a statistically significant ($P < 0.05$) increase (~1.8-, 4.7-, 4.6- and 7.7-fold, respectively) in the levels of tyrosine-phosphorylated erbB1 (Figure 2B). Immunoblotting the membrane with anti-erbB1 antibody (Figure 2A, lower panel) and densitometric analysis of the blots (data not shown) did not reveal any differences in erbB1 protein following different treatments. These results suggested that the observed increase in erbB1 activation by IP6 was not due to an increase in erbB1 protein. Treatment of starved cultures with IP6 alone at 1 mM did not affect tyrosine phosphorylation or the level of erbB1 protein (data not shown), suggesting that the observed increase in erbB1 activation following treatment with IP6 plus ligand is not due to a direct receptor activation by IP6 alone. When erbB1 activation data were compared with the binding of erbB1 to AP2, they were inversely related (compare Figure 2 with Figure 1). This suggests that the observed increase in erbB1 activation by IP6 could have been due to impairment of erbB1 endocytosis where a lack of binding with AP2 leaves the activated erbB1 receptor at the cell surface instead of being internalized and degraded by a stepwise process (22,23). These results also suggest that IP6 impairs receptor endocytosis by targeting its effect on AP2 binding with erbB1 in an erbB1 activation-independent manner.

IP6 inhibits Shc activation and Shc–erbB1 binding in DU145 cells

We next assessed the effect of erbB1 endocytosis impairment by IP6 on the erbB1-mediated immediate downstream signaling pathway involving Shc activation. In contrast to erbB1 activation data, treatment of cultures with different concentrations of IP6 for 2 h, before the addition of TGFα, showed a strong decrease in the tyrosine phosphorylation of Shc protein (Figure 3A, lanes 3–6). As a control, serum starvation of cells for 36 h led to complete absence of tyrosine phosphorylated 46 and 52 kDa Shc proteins (Figure 3A, lane 1); however, treatment of starved cultures with TGFα showed strong phosphorylation of the 46 kDa Shc protein and weak phosphorylation of the 52 kDa Shc protein as evidenced by a reactivity of immunoprecipitated Shc to anti-phosphotyrosine antibodies (Figure 3A, lane 2). Densitometric analysis of the blots from three independent experiments showed that the levels of phosphorylated 52 kDa Shc protein were changed inconsistently following different treatments (data not shown). However, in the case of the phosphorylated 42 kDa Shc protein, 0.5, 1 and 2 mM IP6 pretreatment followed by ligand, resulted in a statistically significant ($P < 0.05$) decrease (77–84%) in its levels compared with treatment with ligand alone (Figure 3D). The observed inhibitory effect of IP6 on Shc activation was not due to a change in Shc protein levels (Figure 3B). Shc proteins contain a Src homology 2 (SH2) domain, which binds to phosphotyrosine-containing sequences, including erbB1, upon ligand activation. As shown in Figure 3C, there was strong binding in the sample treated with TGFα alone (lane 2), whereas IP6 treatment at various concentrations resulted in a very strong decrease in the binding of Shc to erbB1 (Figure 3C, lanes 3–6). Densitometric analysis of the blots from three independent studies showed that, compared with ligand alone, IP6 treatments resulted in a statistically significant decrease (58–100%; $P < 0.05$) in the binding of Shc to erbB1 (Figure 3D). This observation further suggests an inhibition of erbB1-mediated downstream signaling following impairment of ligand-activated erbB1 binding to AP2 and thereby inhibition of receptor endocytosis by IP6. Studies are in progress to test this hypothesis and rule out any possibility that IP6 has a direct effect on Shc phosphorylation independent of an inhibitory effect on activated erbB1 endocytosis.

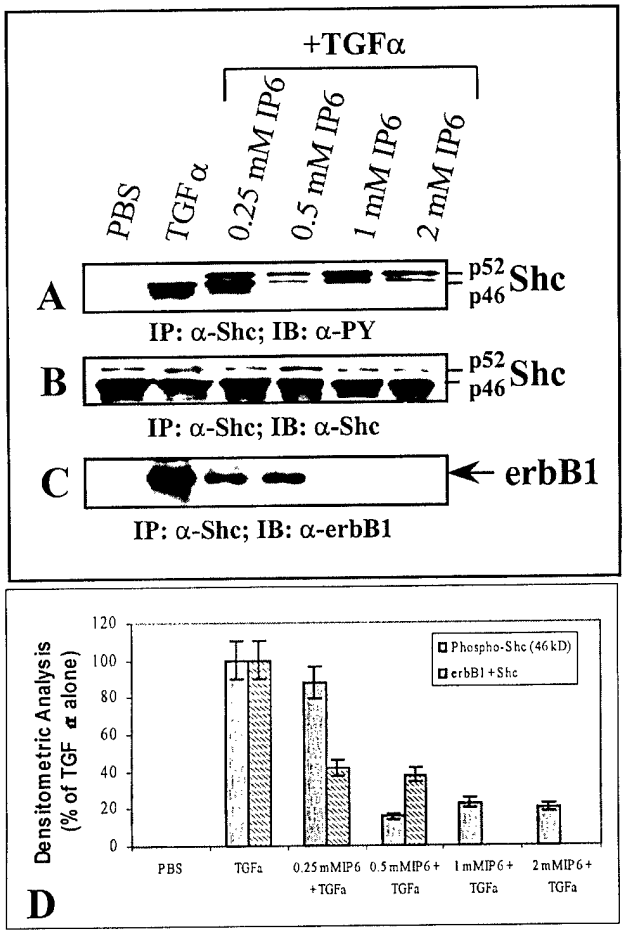

Fig. 3. Impairment of erbB1 receptor-mediated endocytosis by IP6 inhibits erbB1-mediated mitogenic signaling by inhibiting Shc activation and Shc–erbB1 binding in DU145 cells. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. Shc was immunoprecipitated using an anti-Shc antibody and immunoprecipitates were subjected to SDS–PAGE followed by western blotting. The membrane was probed with anti-phosphotyrosine (**A**), anti-Shc (**B**) or anti-erbB1 (**C**) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualized using the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (**D**) Densitometric analysis data (mean ± SD) from three independent experiments.

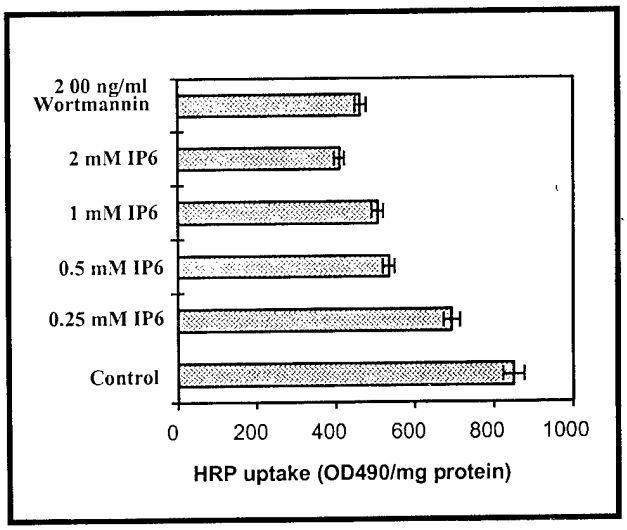

Fig. 4. IP6 also impairs fluid-phase endocytosis in DU145 cells. Cultures in 35 mm dishes at 70–80% confluence were washed with α-MEM three times and treated with 0, 0.25, 0.5, 1 or 2 mM IP6 or 200 ng/ml wortmannin in α-MEM for 2 h at 37°C. Next, 2 mg/ml HRP and 1% bovine serum albumin were added and cultures were incubated for another hour at 37°C. HRP uptake was determined as detailed in Materials and methods. The data shown are mean ± SE of three independent experiments, each done in duplicate.

IP6 also inhibits fluid-phase endocytosis in DU145 cells

To determine the effect of IP6 on fluid-phase endocytosis in DU145 cells, HRP uptake experiments were carried out as described (60). As shown in Figure 4, compared with control, IP6 treatment resulted in a dose-dependent decrease in HRP uptake in DU145 cells as measured by HRP activity in terms of its binding with cellular proteins (60). The lowest concentration (0.25 mM) of IP6 used in this study caused only 19% inhibition ($P < 0.05$) of fluid-phase endocytosis (Figure 4). Much higher inhibition was observed at 0.5, 1 and 2 mM IP6, accounting for 38%, 42% and 52% inhibition ($P < 0.05$), respectively (Figure 4). Treatment of cells with a PI3K inhibitor, wortmannin, also showed similar decrease in fluid-phase endocytosis (Figure 4; 44% inhibition, $P < 0.05$). From these results with wortmannin, showing inhibition of fluid-phase endocytosis, as compared with the findings that it did not change the binding of AP2 to erbB1 in the receptor-mediated endocytosis study (Figure 1A, lane 6), it can be concluded that fluid-phase endocytosis involves a PI3K-mediated pathway in DU145 cells, whereas receptor-mediated endocytosis does not.

Inhibition of fluid-phase endocytosis by IP6 is mediated by impairment of the PI3K–AKT pathway in DU145 cells

As IP6 and the PI3K inhibitor wortmannin inhibited fluid-phase endocytosis, further studies were performed to delineate the involvement of PI3K–AKT pathway in this process. DU145 cells serum starved for 36 h did not show any tyrosine phosphorylation band for the 110 or 85 kDa PI3K subunits (Figure 5A, top panel, lane 1). However, immunoprecipitation of PI3K and blotting with an anti-phosphotyrosine antibody clearly showed that treatment of serum-starved cultures with TGFα results in a marked tyrosine phosphorylation of 110 kDa PI3K protein (Figure 5A, top panel, lane 2). We also detected tyrosine phosphorylation of a band at ~170 kDa in this sample (Figure 5A, lane 2). This band could be an erbB family member since activated erbB receptors have been shown to bind and activate PI3K (19–22, 31–35). Studies are in progress to characterize this band further. In the studies assessing the effect of IP6 on PI3K phosphorylation, pretreatment of cells with IP6 resulted in a very strong inhibition of ligand-induced tyrosine phosphorylation of the 110 kDa PI3K band (and the ~170 kDa protein) (Figure 5A, top panel, lanes 3–5). Densitometric analysis (Figure 5B) of the blots from three independent studies showed that IP6 caused a statistically significant decrease (56%; $P < 0.05$) in tyrosine phosphorylation of the 110 kDa PI3K band at 0.25 mM and 100% inhibition ($P < 0.05$) at ≥0.5 mM (Figure 5A, top panel, lanes 4 and 5; Figure 5B). Wortmannin (at 200 ng/ml) also completely inhibited tyrosine phosphorylation of the 110 kDa PI3K band (Figure 5A, top panel, lane 6; Figure 5B). The observed activation of the 110 kDa PI3K band by TGFα and its inhibition by IP6 was not due to a change in either the amounts of the 110 and 85 kDa PI3K proteins (Figure 5A, bottom panel).

AKT, a downstream target of PI3K, becomes serine–threo-

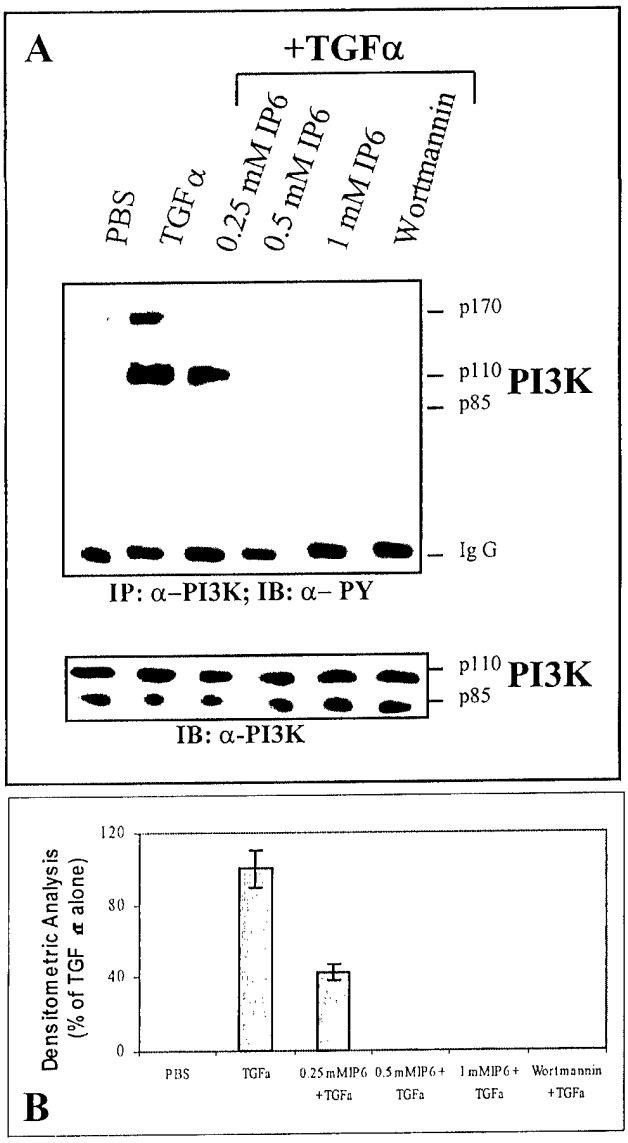

Fig. 5. Inhibition of fluid-phase endocytosis by IP6 is mediated by impairment of the PI3K–AKT pathway in DU145 cells: effect on PI3K activation. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. PI3K was immunoprecipitated using an anti-PI3K antibody, and immunoprecipitates or total cell lysates were then subjected to SDS–PAGE followed by western blotting as described in Materials and methods. (**A**) Immunoprecipitated PI3K were blotted and then probed with an anti-phosphotyrosine antibody (upper panel), and cell lysates were blotted and then probed with an anti-PI3K (lower panel) antibody. Membranes were then incubated with a peroxidase-conjugated appropriate secondary antibody and visualized using the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (**B**) Densitometric analysis data (mean ± SD) from three independent experiments.

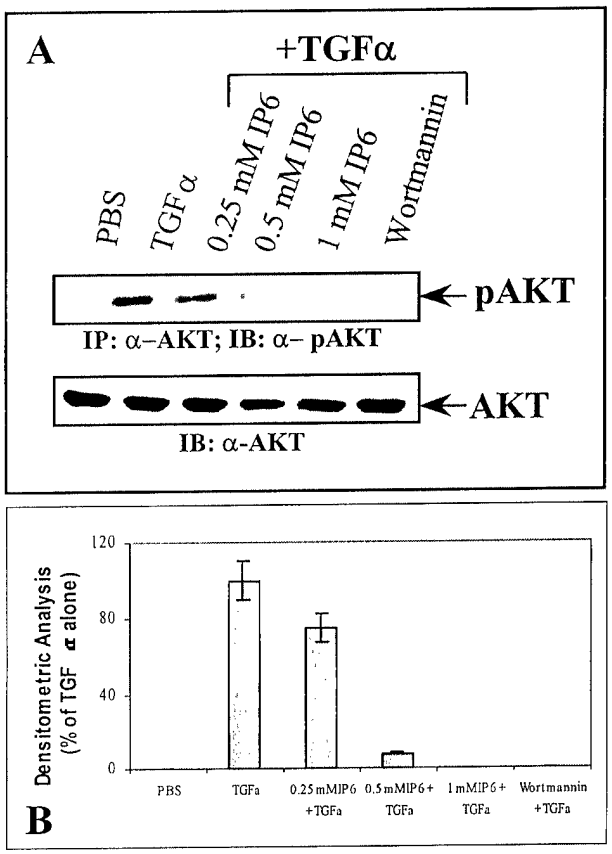

Fig. 6. Inhibition of fluid-phase endocytosis by phytic acid is mediated by impairment of PI3K-AKT pathway in DU145 cells: effect on AKT activation. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. AKT was immunoprecipitated using an anti-AKT antibody and immunoprecipitates or total cell lysates were then subjected to SDS–PAGE followed by western blotting as described in Materials and methods. (**A**) After blotting, immunoprecipitated AKT was probed with an anti-phospho-AKT antibody (upper panel), and cell lysates with an anti-AKT antibody (lower panel). Membranes were then incubated with an appropriate peroxidase-conjugated secondary antibody and visualized using the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (**B**) Densitometric analysis data (mean ± SD) from three independent experiments.

nine phosphorylated *in vivo* in a PI3K-sensitive manner (35,61,62). It has been linked to diverse cellular processes including cell survival by suppressing apoptosis via phosphorylation of BAD (63). Based on our data showing that IP6 inhibits ligand-induced activation of PI3K, we next assessed its effect on AKT phosphorylation. As shown in Figure 6A (top panel), immunoprecipitated samples from serum-starved cells showed no reactivity towards an antibody against phospho-AKT, whereas treatment of starved cultures with TGFα resulted in a strong activation of AKT (lanes 1 and 2, respectively). Pretreatment of cells with IP6 resulted in strong to complete inhibition of TGFα-induced AKT activation (Figure 6A, top panel, lanes 3–5). The PI3K inhibitor wortmannin completely inhibited ligand-induced AKT activation (Figure 6A, top panel, lane 6). Densitometric analysis of the blots (Figure 6B) from three independent studies showed that IP6 inhibited on ligand-induced AKT phosphorylation (25–100% decrease in the levels of phospho-AKT; Figure 6B) was statistically significant ($P < 0.05$). The observed changes in the levels of activated AKT were not due to a change in AKT protein levels in different treatment samples (Figure 6A, bottom panel).

IP6 also inhibits MAPK/ERK1/2 phosphorylation in DU145 cells

Via several different cytoplasmic signaling pathways, activation of erbB1 ultimately activates MAPK/ERK1/2, which localizes to nucleus and activates transcription factors for cell growth and proliferation (19–22). Besides inhibiting Shc activation, Shc binding to erbB1 and PI3K–AKT activation, IP6

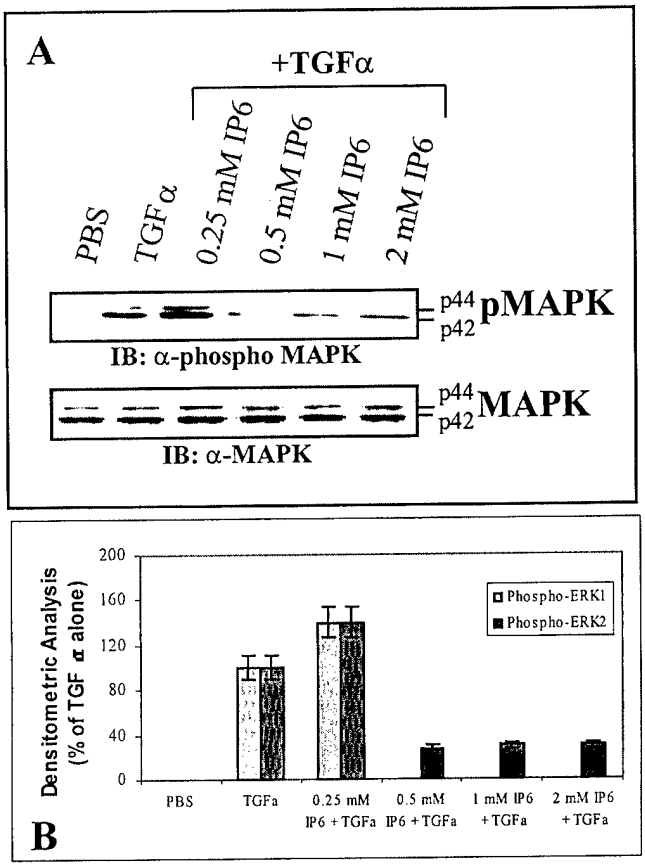

Fig. 7. Inhibition of erbB1 receptor-mediated and fluid-phase endocytosis by IP6 impairs MAPK/ERK1/2 activation in DU145 cells. Treatment of cells and lysate preparations were similar to those summarized in Figure 1 and detailed in Materials and methods. Total cell lysates were subjected to SDS–PAGE followed by western blotting as described in Materials and methods. (**A**) Membranes were probed with anti-phospho-MAPK (upper panel) or anti-MAPK (lower panel) antibody. They were then incubated with an appropriate peroxidase-conjugated secondary antibody and visualized by the ECL detection system. The treatment in each lane is as marked in the figure. In each case, a representative blot is shown from three independent studies with reproducible findings. (B) Densitometric analysis data (mean ± SD) from three independent experiments.

also inhibited TGFα-induced MAPK/ERK1/2 phosphorylation with no change in protein levels (Figure 7A). Densitometric analysis of the blots (Figure 7B) from three independent studies showed that 0.25 mM IP6 resulted in a ~40% increase in phospho-ERK1/2 levels. Higher concentrations of IP6 (0.5, 1 and 2 mM) resulted in 100% inhibition ($P < 0.05$) of ligand-induced ERK1 phosphorylation and 70% inhibition ($P < 0.05$) of ERK2 phosphorylation (Figure 7B). Together, these data provide convincing evidence that IP6 treatment of DU145 cells results in the impairment of receptor-mediated and fluid-phase endocytosis and associated signaling which leads to the inhibition of MAPK signaling pathway as a downstream effect.

IP6 inhibits both anchorage-dependent and -independent growth of DU145 cells

To assess whether impairment by IP6 of erbB1 endocytosis and the mitogenic signaling associated with it, produces biological effects that occur at similar concentrations, we next assessed the effect of IP6 on anchorage-dependent and -independent growth of DU145 cells. As shown by data in Figure 8A, the treatment of cells with IP6 resulted in a significant inhibition of anchorage-dependent cell growth in both concentration- and

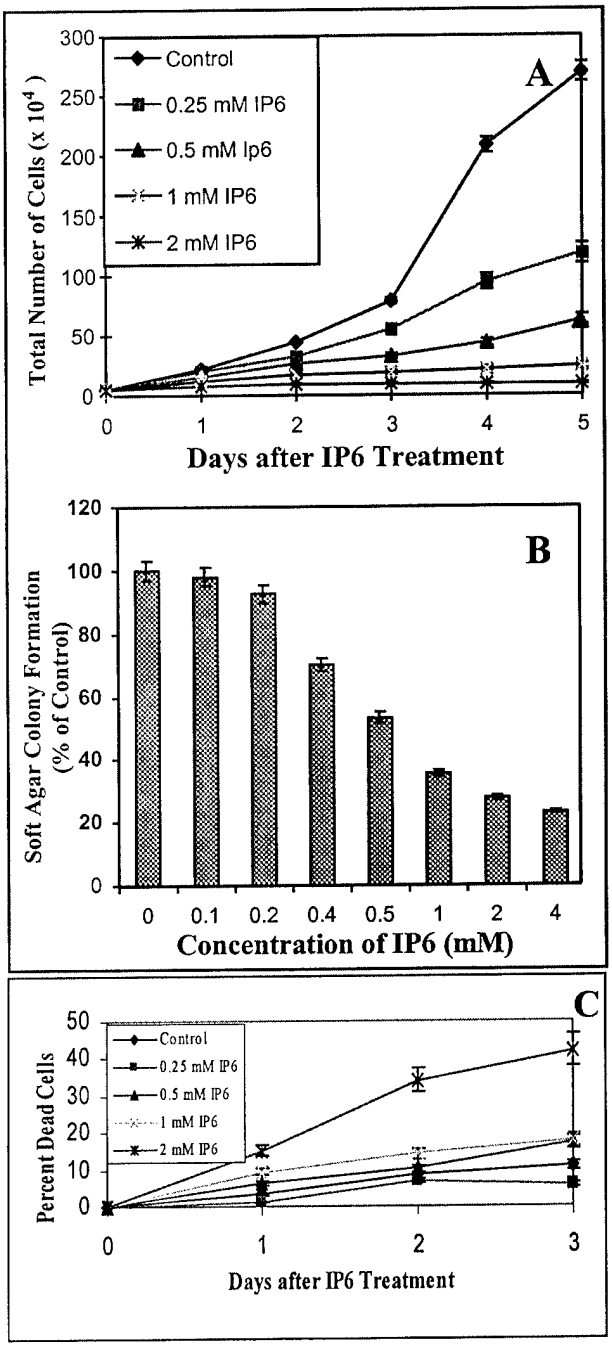

Fig. 8. IP6 inhibits both anchorage-dependent and -independent growth of DU145 cells. To monitor anchorage-dependent cell growth (**A**) and cell death (**C**), cells were plated at 0.5×10^5 cells per 60 mm plate and, on day 2, treated with water or the indicated concentrations of IP6. The total number of cells was counted for varying time periods and cell viability was assessed by Trypan Blue dye exclusion assay. The cell growth data shown are mean ± SE of four independent plates; each sample counted in duplicate. The cell death data shown are mean ± SE of three independent plates; each sample counted in duplicate. Representative data from one experiment are shown; data from three independent studies were reproducible. To investigate anchorage-independent cell growth (**B**), a soft agar colony formation assay was performed using six-well plates as detailed in Materials and methods. The number of colonies was determined under an inverted phase-contrast microscope at ×100 magnification; a group of >16 cells was counted as a colony. The data shown are means ± SE of three independent wells at optimum time (10 days) from the start of cell seeding; the experiment was repeated once with similar results.

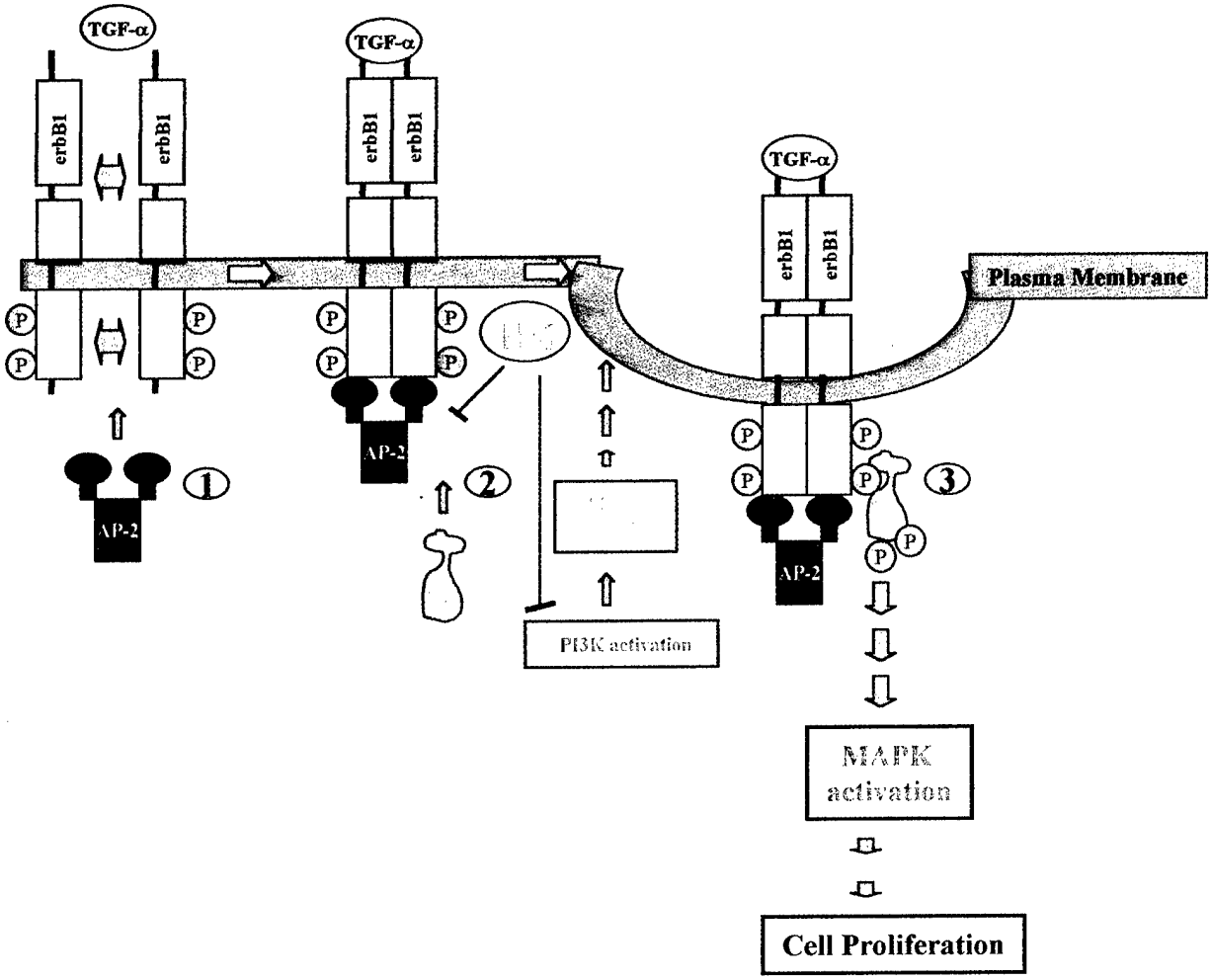

Fig. 9. Proposed mechanism for anti-proliferative action of IP6 on DU145 cells. IP6 impairs TGFα-induced erbB1 receptor endocytosis leading to inhibition of cell proliferation by two mechanisms: (i) by inhibiting erbB1 recruitment of AP2 and Shc and (ii) by inhibiting PI3K–AKT activation. These events result in impairment of both receptor-mediated and fluid-phase endocytosis, and inhibition of mitogenic signaling such as MAPK/ERK1/2 activation, explaining the anti-proliferative action of IP6 in DU145 cells. The numbers shown in black as 1, 2 and 3 represent the steps for erbB1 endocytosis, and associated signaling pathways and cell proliferation.

time-dependent manner. Compared with controls, treatment of cells with 0.25 mM IP6 showed >50% inhibition (statistically significant; $P < 0.05$) of cell growth at day 4 of treatment (Figure 8A). Much greater inhibition of cell growth occurred with 0.5 mM IP6 throughout the treatment time; by day 4 of treatment there was almost 80% inhibition ($P < 0.05$) (Figure 8A). At 1 and 2 mM IP6, no cell growth was observed after 1 day of treatment throughout the study (Figure 8A).

We next assessed the effect of IP6 on anchorage-independent growth of DU145 cells using soft agar colony formation assay. As many as 54.5 ± 3.6 (mean ± SE of three independent plates) colonies/1000 cells (per plate) were counted in controls after 10 days of initial seeding (data not shown). Treatment of cells with IP6 resulted in a concentration-dependent inhibition in soft agar colony formation by DU145 cells (Figure 8B). The lower concentrations of IP6 (0.1 and 0.2 mM) showed almost no inhibition, whereas ~30% and 50% inhibition (statistically significant; $P < 0.05$) was evident at 0.4 and 0.5 mM, respectively (Figure 8B). The highest concentration of IP6 (4 mM) used in this assay caused a reduction by almost 75% (statistically significant; $P < 0.05$) in the number of colonies per plate.

Trypan Blue exclusion assays were used to assess the viability of IP6-treated cells; as shown in Figure 8C, 0.5, 1 and 2 mM IP6 caused statistically significant ($P < 0.05$) cell death, compared with controls, accounting for 6–40% death in a concentration- and time-dependent manner. These data suggest that the observed growth-inhibitory effects of IP6, shown in Figure 8A and B, could be due to its inhibitory effects on mitogenic signaling as well as cytotoxicity. Studies are currently in progress to assess and define apoptotic effects of IP6 in human prostate carcinoma cells.

Discussion

As summarized in Figure 9, the central finding of the present study is that IP6 impairs both receptor-mediated and fluid-phase endocytosis, resulting in the inhibition of mitogenic signals associated with growth and proliferation of human prostate carcinoma DU145 cells. The results obtained suggest a novel molecular pathway to be further explored for the intervention of advanced and androgen-independent human PCA by IP6. The major thrust in PCA control has been to design and develop intervention approaches based on molecular mechanisms (14,15). For example, the major emphasis of the National Cancer Institute, NIH, has been to develop surrogate

endpoint biomarkers for early detection, risk assessment and treatment of PCA (14,15). Special emphasis has also been placed on those markers which relate to the progression of microscopic to clinically relevant PCA that could be explored in intervention trials (14,15). As found in a significant number of routine needle biopsies without cancer, high-grade PIN is the most likely precursor of PCA, so PIN has been extensively used as a suitable endpoint biomarker for PCA treatment in clinical trials (14,15). It is important to emphasize here that erbB family members are one of the few potential surrogate endpoint genetic markers which are being employed for the screening of agents for treating PCA in short-term phase II clinical trials (14–18). ErbB family members are also being extensively explored as potential molecular targets for PCA intervention, specifically in the case of androgen-independent PCA (20). In view of these efforts, the results of the present study showing that a naturally occurring phytochemical, IP6, impairs erbB1 receptor endocytosis and associated mitogenic signaling in advanced and androgen-independent human PCA DU145 cells, could have direct implications in the treatment of advanced and androgen-independent human PCA.

The erbB and other receptor- and non-receptor-mediated signaling cascades activate MAPKs, a family of signaling molecules which are the ultimate cytoplasmic targets in signaling cascades (reviewed in refs 64–68). Following their activation, MAPKs are translocated to the nucleus where they activate transcription factors for cell growth, proliferation and differentiation (64–68). These studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, it has been shown recently that MAPK/ERK1/2 is constitutively active in human PCA DU145 cells (69). This study also showed that epidermal growth factor, insulin like growth factor 1 (IGF-1) and protein kinase A activator significantly activate MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells via the erbB1 receptor (69). An increase in the activation of MAPK/ERK1/2 signaling has also been reported recently as human PCA progresses to a more advanced and androgen-independent malignancy (70). Consistent with the involvement of activated MAPK/ERK1/2, possibly via an erbB1 autocrine loop, in the progression of advanced and androgen-independent human PCA, in the present study, we observed that impairment of erbB1 endocytosis by IP6 also results in the inhibition of MAPK/ERK1/2 activation in DU145 cells (Figure 9). The observed inhibitory effect of IP6 on MAPK/ERK1/2 activation could be via impairment of erbB1–Shc–Ras/Raf and/or erbB1–PI3K pathways (Figure 9); more studies are in progress to define further the specific signaling pathway affected by IP6 in inhibiting MAPK/ERK1/2 activation. The specificity of IP6 in inhibiting PI3K followed by AKT activation and fluid-phase endocytosis also needs to be further explored. For example, whereas it can be argued that the inhibitory effect of IP6 on PI3K activation observed in the present study is due to the impairment of ligand-induced erbB1 receptor endocytosis in DU145 cells, direct inhibition of PI3K by IP6 has also been reported in a recent *in vitro* assay (57). This study (57) also showed that IP6 significantly inhibits tumor promoter-induced cell transformation, AP-1 activity, PI3K activity and MAPK/ERK1/2 activation in JB6 cells. Consistent with the effects of IP6 reported in previous study (57), we have identified additional upstream molecular signaling events which are impaired by IP6 as a plausible cause for its downstream inhibition of PI3K and MAPK/ERK1/2 followed by AP-1 activation and cell transformation (this study).

The results obtained in the present study for the activation of the 110 kDa PI3K subunit by TGFα differ from those observed by us in another study showing that treatment of serum-starved DU145 cells with IGF-1 or neu differentiation factor (NDF) results in the activation of the 85 kDa PI3K subunit (36). PI3K can be activated by (i) its 85 kDa subunit, which binds to tyrosine kinase autophosphorylated at the sequence YXXM, a specific SH2 domain phosphotyrosine-binding sequence or (ii) its 110 kDa subunit, which binds to the Ras effector domain in a GTP-dependent manner (33,61). Differential ligand-induced activation of PI3K 110 and 85 kDa subunits observed by us in a recent study (36) suggests that, in DU145 cells, TGFα activates the PI3K class which does not interact with SH2 domain-containing adaptors but contains an amino-terminal Ras-binding site and therefore interacts with Ras proteins in a GTP-dependent manner. More detailed mechanistic studies are in progress to address this pathway further. However, to our knowledge, ours is the first report showing that TGFα activates the 110 kDa subunit of PI3K in DU145 cells and that IP6 inhibits this activation.

Several recent studies have shown a direct involvement of the PI3K–AKT pathway in fluid-phase endocytosis by regulating Rab5, which is active in its GTP-bound form and is a rate-limiting factor for endocytosis (33,34); specifically, Ras–PI3K is connected to the activation of AKT, which is a key regulator of fluid-phase endocytosis (33,34). In accordance with our present finding that TGFα activates the 110 kDa PI3K subunit in DU145 cells, as compared with the 85 kDa PI3K subunit by IGF-1 and NDF found in another study by us (36), TGFα also showed strongest effect in terms of fluid-phase endocytosis of HRP compared with that by IGF-1 and NDF (36). Together, these results further support the arguments that the Ras–PI3K–AKT pathway is involved in fluid-phase endocytosis and that IP6 inhibits fluid-phase endocytosis via inhibition of the PI3K–AKT pathway (Figure 9).

Based on the data reported here, together with earlier findings showing that IP6 has cancer-preventive and anti-carcinogenic effects in several tumor models, we suggest that the role of dietery IP6 in preventing human PCA be explored. Diet varies significantly from country to country, and has been estimated to account for up to 35% of differences in overall cancer rates (71). For example, the incidence of prostate, breast and colon cancers is lower in Asian countries than in the West, including the USA; people living in Japan, China, Korea and other Asian countries are four to 10 times less likely to be diagnosed with and die from prostate and breast cancers than those in the USA (1). In the specific case of PCA, the clinical incidence of malignancy is low in Asian men and highest in African-Americans and Scandinavians (1,72). However, the incidence and mortality rate due to PCA in Asian men who have moved to the USA are approximately the same as those in Americans (72). Epidemiological studies suggest that dietary and environmental factors are the major causes of increase in PCA in the USA men as well as in migrating Asians (1,72). Low-fat and/or high-fiber diets significantly affect sex hormone metabolism in men (73), so despite the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and the mortality rate associated with it is low in Japan and some other Asian countries (72). This could, at least partly, be explained by a diet-related reduction in this malignancy (73,74). IP6 is also a ubiquitous plant component

constituting 0.4–6.4% of most cereals, nuts, legumes, oil seeds and soybean, and its levels are high in certain dietary fibers. Diets rich in IP6 could be beneficial for preventing PCA in particular and other human malignancies in general.

Acknowledgement

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Appendix 7

The Prostate 46:98–107 (2001)

Detrimental Effect of Cancer Preventive Phytochemicals Silymarin, Genistein and Epigallocatechin 3-gallate on Epigenetic Events in Human Prostate Carcinoma DU145 Cells

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BACKGROUND. Targeting epigenetic events associated with autonomous growth of advanced prostate cancer (PCA) is a practical approach for its control, prevention, and treatment. Recently we showed that treatment of prostate carcinoma DU145 cells with cancer preventive flavonoid silymarin at 100–200 μM doses inhibits erbB1-Shc mitogenic signaling and modulates cell cycle regulators leading to a G_1 arrest and inhibition of cell growth and anchorage-independent colony formation. Here, we asked the question whether these important findings could be extended to other cancer preventive flavonoids and isoflavones such as epigallocatechin 3-gallate (EGCG) and genistein.

METHODS. DU145 cells were treated with similar doses (100–200 μM) of silymarin, genistein or EGCG, cell lysates prepared, and levels of activated signaling molecules (erbB1-Shc-ERK1/2) and cell cycle regulators (CDKIs, CDKs, and cyclins) analyzed employing immunoprecipitation and/or immunoblotting techniques. Cell growth studies were done by cell counting during 5 days of treatment with these agents, and cell death was determined by Trypan blue staining.

RESULTS. Treatment of cells with silymarin, genistein or EGCG at 100–200 μM resulted in a complete inhibition of TGFα-caused activation of erbB1 followed by a moderate to strong inhibition (10–90%) of Shc activation without an alteration in their protein levels. Silymarin and genistein, but not EGCG, also inhibited (10% to complete) ERK1/2 activation suggesting that these agents impair erbB1-Shc-ERK1/2 signaling in DU145 cells. In other studies, silymarin, genistein or EGCG caused a strong induction of Cip1/p21 (up to 2.4-fold) and Kip1/p27 (up to 150-fold), and a strong decrease in CDK4 (40–90%) but had moderate effect on CDK2, and cyclins D1 and E. An enhanced level of CDKIs also led to an increase in their binding to CDK4 and CDK2. Treatment of cells with silymarin, genistein or EGCG also resulted in 50–80% cell growth inhibition at lower doses, and complete inhibition at higher doses. In contrast to silymarin, higher doses of genistein showed cytotoxic effect causing 30–40% cell death. A more profound cytotoxic effect was observed with EGCG accounting for 50% cell death at lower doses and complete loss of viability at higher doses.

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CONCLUSION. These results suggest that similar to silymarin, genistein and EGCG also inhibit mitogenic signaling pathway(s) and alter cell cycle regulators, albeit at different levels, leading to growth inhibition and death of advanced and androgen-independent prostate carcinoma cells. More studies are, therefore, needed with these agents to explore their anti-carcinogenic potential against human prostate cancer. *Prostate 46:98–107, 2001.*

KEY WORDS: **EGFR; MAPK; cell cycle; silymarin; genistein; EGCG**

INTRODUCTION

Prostate cancer (PCA) is the most common invasive malignancy and second (to lung cancer) leading cause of cancer mortality in American men [1]. Progression of PCA depends on both genetic and epigenetic factors. The multi-step process of PCA progression leads from transformation over an androgen-dependent non-metastatic phenotype to a more malignant metastatic androgen-independent phenotype [2]. Receptor tyrosine kinases (RTKs) are the major components of signal transduction pathways that play an essential role in cell growth, proliferation, and differentiation [3]. However, enhanced tyrosine kinase activity due to overexpression of RTKs or non-RTKs, leads to persistent mitogenic signaling, and has been established as a major contributor to carcinogenesis [3]. In human PCA, RTKs and associated mitogenic and anti-apoptotic signaling have been shown to be both genetic and epigenetic causes for disease progression [4–10]. For example, an enhanced expression of epidermal growth factor receptor (EGFR or erbB) family members (erbB1/EGFR, erbB2, erbB3) and associated ligands has been shown to be a causal genetic event in advanced and androgen-independent PCA growth and metastasis [4–7]. These genetic alterations lead to an epigenetic feedback event where ligand (e.g., transforming growth factor α (TGFα)/epidermal growth factor (EGF)) and receptor (e.g. erbB1) make a functional autocrine loop, thus facilitating hormone-independent uncontrolled growth of PCA [8–10].

Together, it can be appreciated that possibly other than gene therapy, it is difficult to fix genetic alterations for the control of advanced PCA. Therefore, targeting epigenetic events such as impairment of tyrosine kinase activity and associated mitogenic signaling pathway(s) could be a practical and translational approach to control advanced and androgen-independent PCA, and to prevent and treat the disease from further progression. Fruits, vegetables, common beverages, and several herbs and plants with diversified pharmacological properties have been shown to be rich sources of micro-chemicals with cancer preventive effects in humans [11,12]. Among these, naturally occurring flavonoids and isoflavones have been receiving increasing attention in recent years [13–15]. Accordingly, the major objective of present study was to assess the effect of cancer preventive phytochemicals silymarin (flavanone), genistein (isoflavone), and epigallocatechin 3-gallate (EGCG) (flavanol) on epigenetic events involved in uncontrolled growth of advanced and androgen-independent PCA.

Silymarin is a dietary supplement present in milk thistle (*Silybum marianum*) seeds, and used clinically in Europe, Asia, and the United States for the treatment of liver disease [16]. In recent years, several studies by us have shown the cancer preventive effects of silymarin in skin tumorigenesis models and its anti-carcinogenic activity in human prostate, breast, and cervical carcinoma cells [16–19]. Genistein is a dietary agent present in soybeans, and has received much attention as a potential anti-carcinogenic agent due to its effect on a number of cellular processes [20,21]. Several epidemiological and animal tumor studies have shown the preventive effects of genistein against various cancers [15,22]. With regard to PCA, the anti-carcinogenic and cancer preventive effects of genistein are well studied using cell and organ cultures, and animal models [23–25]. The efficacy of genistein is also being evaluated in PCA patients [26]. Tea (*Camellia sinensis*) is one of the most common beverages all over the world. Several studies from our group and by others have shown the cancer preventive and anti-carcinogenic effects of tea polyphenols on various cancers including skin, lung, esophagus, stomach, liver, intestine, pancreas, breast, and prostate [14,27]. As a major component, EGCG constitutes ~50% (w/w) of the total green tea extract, and is attributed for both cancer preventive and anti-carcinogenic effects of green tea [14,27–30].

Based on the above findings, in the present study, our major goal was to assess the effect of silymarin, genistein, and EGCG on erbB1-Shc-ERK1/2-mediated mitogenic signaling and modulation of cell cycle regulators in androgen-independent human prostate carcinoma DU145 cells. The rationale for these studies was also based on our recent findings where we showed that treatment of DU145 cells with silymarin at

100–200 μM doses inhibits erbB1-Shc mitogenic signaling and modulates cell cycle regulators leading to a G_1 arrest and inhibition of cell growth and anchorage-independent colony formation [18]. Accordingly, our other goal was to determine whether these important findings with silymarin could be extended to other cancer preventive flavonoids and isoflavones such as EGCG and genistein.

MATERIALS AND METHODS

Materials

DU145 cells were from American Type Culture Collection (Manassas, VA); and RPMI-1640 medium, fetal bovine serum, penicillin–streptomycin, TGFα, and all other cell culture reagents were from Life Technologies (Gaithersburg, MD). Silymarin, genistein, and EGCG were from Sigma-Aldrich (St. Louis, MO). Antibodies to EGFR, Shc, and phosphotyrosine were from Upstate Biotechnology (Waltham, MA), and antibodies against CDK2, Cyclin D1, Cyclin E, rabbit anti-mouse IgG, and goat-anti-rabbit IgG-horse radish peroxidase conjugated secondary antibodies and protein A/G agarose beads were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Cip1/p21 was from Calbiochem (Cambridge, MA), and anti-Kip1/p27 and anti-CDK4 antibodies were from Neomarkers Inc. (Fremont, CA). Phospho- (and regular) MAPK/ERK1/2 antibodies were from New England Biolabs (Beverly, MA). ECL detection system was from Amersham Corp. (Arlington Heights, IL).

Cell Culture and Treatments

DU145 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in an atmosphere of 95% air and 5% CO_2 under 90–95% humidity. For erbB1-mediated signaling studies, cells were grown to 70–80% confluency, and then serum-starved for 48 hr. During the final 2 hr of starvation, cells were treated with ethanol or 100–200 μM doses of silymarin, genistein or EGCG in ethanol. The selection of these doses was based on our recent study with silymarin at identical doses [18]. The final concentration of ethanol in the culture medium during the treatment with agents was 0.5% (v/v) and, therefore, the same concentration of ethanol was present in control dishes. Cells were then treated with PBS or TGFα at a concentration of 100 ng/ml of medium, and incubated for 10 min at 37°C. Thereafter, the medium was removed, cells washed with PBS two times, and cell lysates prepared in non-denaturing lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM PMSF, 0.5% NP-40, and 0.2 U/ml aprotinin) as detailed recently [18]. The lysates were cleared by centrifugation at 10,000 rpm for 15 min in tabletop centrifuge at 4°C, the supernatants were collected, and protein concentration determined [18]. To assess the protein levels of cell cycle regulatory molecules, ~80% confluent cells (without serum starvation) were treated with either ethanol or 100–200 μM doses of silymarin, genistein or EGCG in ethanol for 20 hr and cell lysates prepared [18].

Immunoprecipitation and Western Blotting

Cell lysates (200–400 μg protein lysate per sample) were diluted to 1 ml with lysis buffer, and pre-cleared with 25–30 μl protein A/G agarose beads by gentle rotation at room temperature for 1 hr followed by the removal of beads using 5 min centrifugation at 2,000 rpm. The pre-cleared lysates were incubated overnight at 4°C with continuous rotation with 2 μg primary antibody against erbB1, Shc, Cip1/p21 or Kip1/p27, and 25 μl protein A/G agarose beads. Thereafter, immunocomplexes were collected by centrifugation at 2,000 rpm for 5 min, and washed four times with lysis buffer. For immunoblotting, immunocomplexes or cell lysates (20–80 μg protein) were denatured with sample buffer, the samples were subjected to SDS-PAGE (8/12% gel) and transferred on to nitrocellulose membranes. Membranes were blocked with blocking buffer at room temperature for 1 hr and, as desired, probed with primary antibody against phosphotyrosine, erbB1, Shc, phospho- (or regular) MAPK/ERK1/2, Cip1/p21, Kip1/p27, cyclin D1, cyclin E, CDK2 or CDK4 overnight followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system.

Cell Growth and Viability Assays

DU145 cells were plated at a density of 1×10^5 cells per 60 mm dish, and on the following day (Day 0), fed with fresh medium and treated with ethanol or 50–200 μM doses of silymarin, genistein or EGCG in ethanol. The cultures were fed with a fresh medium with or without the same concentrations of compounds every alternate day up to the end of the experiment. On days 1–5 after these treatments, cells were trypsinized, collected, and counted on a hemocytometer. Trypan blue dye exclusion was used to determine cell death.

Densitometric and Statistical Analysis

Autoradiograms of the Western immunoblots were scanned using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background,

and the mean density for each band was analyzed using Scanimage Program (National Institutes of Health, Bethesda, MD). The densitometric data (arbitrary numbers) are shown under the immunoblots at appropriate places, and are average of three independent experiments with less than ± 10% variation. For ERK1/2 phosphorylation studies, the densitometric analysis data for phospho-ERK1/2 blots were corrected for loading with the density of ERK1/2 blots. The two-tailed Student *t*-test was employed to assess statistical significance of difference between vehicle and agent treated samples. The results shown are representative of three independent experiments with similar findings, unless otherwise specified.

RESULTS

Inhibition of TGFα-Caused erbBI Activation

First we assessed the effect of silymarin, genistein, and EGCG on TGFα-caused erbB1 activation in DU145 cells. A 48-hour serum starvation of cells resulted in a complete disappearance of constitutively active erbB1 [18] as evident by a lack of reactivity of immunoprecipitated erbB1 with anti-phosphotyrosine antibody (Fig. 1A, lane 1). Treatment of serum-starved cells with TGFα, however, resulted in a strong activation of erbB1 (Fig. 1A, lane 2). Pretreatment of cultures with silymarin, genistein, and EGCG during the last 2 hr of serum starvation followed by TGFα stimulation resulted in a complete inhibition of ligand-caused activation of erbB1 (Fig. 1A, lanes 3–11). The densitometric quantification of the bands (Fig. 1A) showed that, compared to TGFα-treated positive control, silymarin treatment at 100 μM dose followed by TGFα stimulation resulted in no effect on erbB1 activation. However, 150 and 200 μM doses of silymarin and all three doses of genistein and EGCG showed complete inhibition ($P < 0.001$, $n = 3$) of ligand-caused erbB1 activation (Fig. 1A, lanes 6–11). The observed inhibitory effect of these agents was not due to a decrease in erbB1 protein levels in all but 100 μM genistein sample (Fig. 1B) suggesting that these agents inhibit TGFα-caused erbB1 activation.

Inhibition of Shc Activation and Its Binding to erbBI

One of the immediate downstream substrates to erbB1 activation is Shc which following tyrosine phosphorylation, acts as an adaptor between erbB1 and other SH2-containing proteins in erbB1-Grb2-SOS/ras/raf/ERK-mediated mitogenic signaling [3,18]. Based on the inhibitory effect of silymarin, genistein or EGCG on erbB1 activation, we next assessed whether these agents also impair Shc activation. As shown in Figure 2A, a pattern parallel to erbB1 activation was also evident for Shc. There was no Shc activation in serum-starved cultures, but treatment of starved cells with TGFα resulted in strong activation of the 46 kDa isoform of Shc and only weak activation of 52 kDa Shc isoform (Fig. 2A, lane 2). However, treatment of cultures with different doses of silymarin, genistein, and EGCG for 2 hr prior to the addition of TGFα showed a moderate to strong decrease in tyrosine phosphorylation of both 52 and 46 kDa Shc protein bands (Fig. 2A, lanes 3–11). Quantification of 46 kDa band intensity showed that silymarin, genistein or EGCG caused 10–60% ($P < 0.1$–0.001), 80–90% ($P < 0.001$), and 70–90% ($P < 0.001$) inhibition (an average of three independent studies ± <10% of average) of Shc activation, respectively. The inhibitory effect of these agents on Shc activation was not due to a decrease in total Shc protein levels (Fig. 2B). Since following erbB1 activation, Shc binds to activated erbB1 that causes Shc activation to transduce downstream signals [3,18], we also assessed the effect of these agents on erbB1–Shc binding. The quantification of the bands in Figure 2C shows that silymarin, genistein or EGCG treatment resulted in 60–80% ($P < 0.001$), 70% to complete ($P < 0.001$), and 10% to complete ($P < 0.1$–0.001) inhibition of Shc binding to erbB1, respectively. Together, these results suggest that as an initial step, these

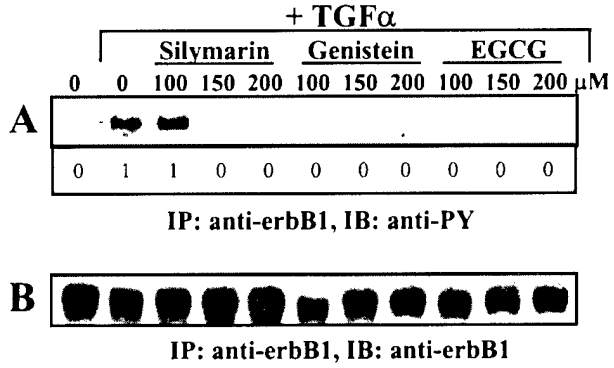

Fig. 1. Silymarin, genistein, EGCG inhibit TGFα-caused activation of erbBI in DUI45 cells. Cells were cultured as described in Methods, and at 70–80% confluency, were serum-starved for 48 hr. During the last 2 hr of starvation, they were treated with ethanol or varying doses of agents in ethanol, and at the end of these treatments with PBS or TGFα (I00 ng/ml of medium) for I5 min at 37°C. Cell lysates were prepared, erbBI was immunoprecipitated using anti-EGFR antibody, and following SDS-PAGE and Western blotting, membranes were probed with (**A**) anti-phosphotyrosine (anti-PY) or (**B**) anti-EGFR (anti-erbBI) antibody, and then peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol + PBS. IP, immunoprecipitation; IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblot in panel A, are average of three independent experiments with less than ± I0% variation.

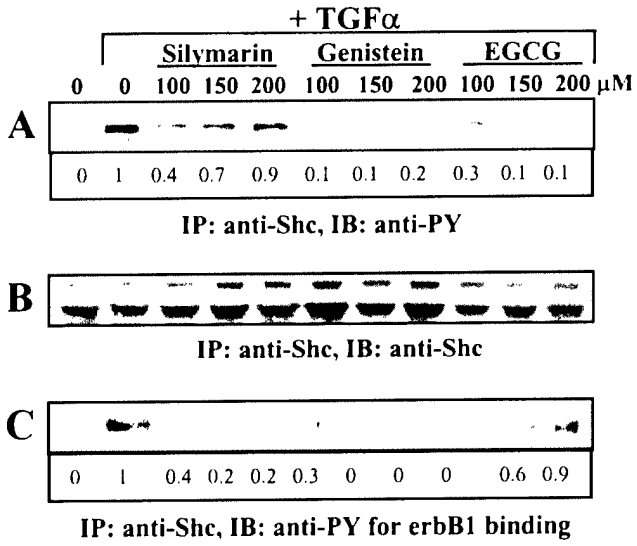

Fig. 2. Silymarin, genistein, and EGCG inhibit Shc activation and its binding with erbBI in DUI45 cells. Cell culture and treatments are those described in Figure I. Shc was immunoprecipitated from the cell lysates using anti-Shc antibody, and following SDS-PAGE and Western blotting, membranes were probed with (**A**) anti-phosphotyrosine (anti-PY), (**B**) anti-Shc or (**C**) anti-phosphotyrosine (anti-PY) for erbBI binding, antibody, and then peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol + PBS. IP, immunoprecipitation; IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in panels A and C, are average of three independent experiments with less than ± I0% variation.

agents inhibit erbB1 activation that leads to a decrease in Shc binding to erbB1 followed by inhibition of Shc activation.

Inhibition of MAPK/ERKI/2 Activation

Based on the data shown in Figures 1 and 2, we then assessed the effect of these agents on the ultimate cytoplasmic mitogenic signaling target, MAPK/ERK1/2 activation. As expected, 48 hr of serum starvation resulted in a completely diminished ERK1/2 activation (Fig. 3A, lane1) while TGFα treatment caused a strong stimulation (Fig. 3A, lane 2). Pretreatment of cultures with silymarin and genistein resulted in a moderate to strong inhibition of ligand-caused activation of ERK1/2 (Fig. 3A, lanes 3–8). Interestingly, EGCG showed a strong increase in phosphorylated levels of ERK1/2 (Fig. 3A, lanes 9–11). The quantification of band intensities for phospho-ERK1/2 and correction for loading with total ERK1/2 showed that silymarin and genistein caused 10–70% ($P < 0.05$–0.001) and 80% to complete ($P < 0.001$) inhibition of phospho-ERK1/2 levels (an average of three independent studies ± <10% of average), respectively. Con-

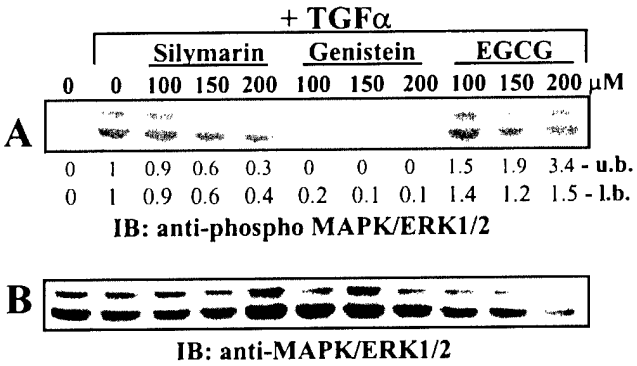

Fig. 3. Silymarin and genistein, but not EGCG, inhibit ligand-caused activation of MAPK/ERKI/2 in DUI45 cells. Cell culture and treatment conditions are described in Methods and Figure I. Cell lysates were subjected to SDS-PAGE and Western blotting, and membranes were probed with (**A**) phospho-MAPK/ERKI/2 or (**B**) MAPK/ERKI/2 antibody. In each case, membranes were then probed with peroxidase-conjugated appropriate secondary antibody. Visualization of proteins was done using the ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol + PBS. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblot in panel A, are average of three independent experiments with less than ± I0% variation, and are corrected for loading with the density of ERKI/2 blots in panel B.

versely, EGCG resulted in 1.2–3.4-fold increase ($P < 0.05$–0.001, n = 3) in phospho-ERK1/2 levels.

Modulation of Cell Cycle Regulators

Based on the observed effect of silymarin, genistein, and EGCG on erbB1-Shc-MAPK/ERK signaling, we assessed the effect of these agents on the modulation of cell cycle regulators. As shown in Figure 4, treatment of cells with these agents resulted in a very strong induction of both Cip1/p21 and Kip1/p27. For Cip1/p21, the most effective agent was silymarin followed by genistein and EGCG showing 1.5–2.4, 1.2–2.2 and 1.7-fold induction ($P < 0.05$–0.001, n = 3), respectively. Only the highest dose of EGCG (200 μM) tested, showed induction of Cip1/p21 whereas lower doses led to a decrease (Fig. 4A). These agents showed 2–120, 70–150, and 15–97-fold increase ($P < 0.001$, n = 3) in Kip1/p27, respectively (Fig. 4B). In other studies, all the test agents exerted a strong decrease in CDK4 protein levels (Fig. 5A). Silymarin was most effective followed by genistein and EGCG accounting for 60–90%, 50–70%, and 40–50% decrease ($P < 0.001$, n = 3). Silymarin was also effective in reducing CDK2 expression by 30% ($P < 0.05$), but genistein and EGCG did not show any effect (Fig. 5B). A moderate inhibitory effect of these agents on cyclin D1 (Fig. 5C) and cyclin E (Fig. 5D) levels was also observed with silymarin being most effective followed by genistein and EGCG.

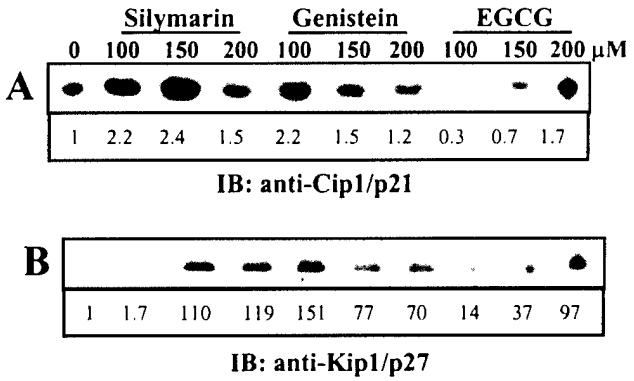

Fig. 4. Silymarin, genistein, and EGCG induce the levels of CDKIs in DU145 cells. Cells were cultured as described in Methods, and at 70–80% confluency (without serum starvation), were treated with either vehicle alone or varying concentrations of silymarin, genistein, and EGCG for 20 hr as described in Methods. At the end of these treatments, total cell lysates were prepared, and subjected to SDS-PAGE followed by Western blotting as described in Methods. Membrane was probed with anti- (**A**) Cipl/p2l or (**B**) Kipl/p27 antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled in the figure; lane 1 labeled as 0 denotes ethanol treatment alone. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in panels A and B, are average of three independent experiments with less than ± 10% variation.

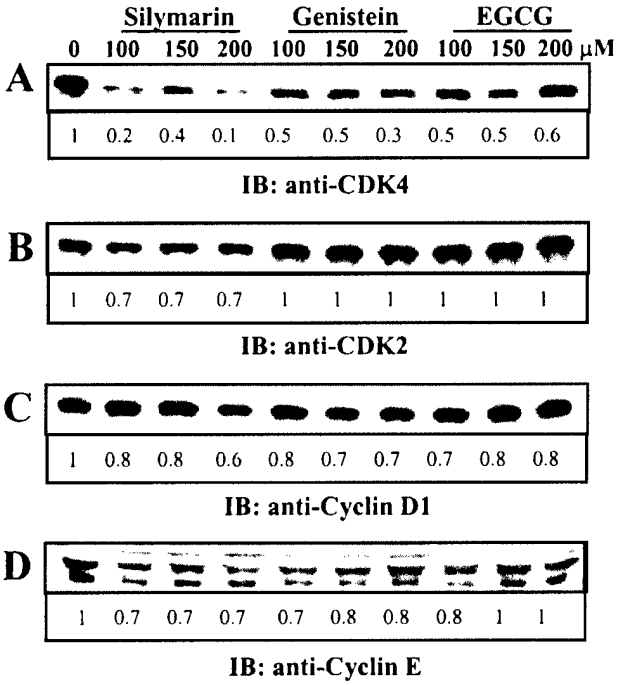

Fig. 5. Effect of silymarin, genistein, and EGCG on CDKs and cyclins in DU145 cells. Cell cultures and treatments are those described in Methods and Figure 4. Total cell lysates were subjected to SDS-PAGE followed by Western blotting as described in Methods. Membrane was probed with anti- (**A**) CDK4, (**B**) CDK2, (**C**) cyclin DI or (**D**) cyclin E antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by the ECL detection system. Different treatments are as labeled in the figure; lane 1 labeled as 0 denotes ethanol treatment alone. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in each panel, are average of three independent experiments with less than ± 10% variation.

To explore the significance of CDKI induction by these agents, we determined the binding of CDKIs to CDKs. As shown in Figure 6A and B, Cip1/p21 binding to CDK4 and CDK2 is significantly ($P < 0.001$) enhanced following treatment with silymarin, genistein, and EGCG. Similar results were also evident in terms of a strong increase ($P < 0.001$) in the binding of Kip1/p27 to CDK4 and CDK2 (Fig. 6C,D). Together, these results suggest that treatment with silymarin, genistein or EGCG leads to perturbations in cell cycle molecules via an increase in CDKI levels. An increased expression of CDKIs led to their increased binding with CDKs that possibly inhibits kinase activity of CDKs and associated cyclins. Together, these alterations in cell cycle regulators lead to inhibition of cell growth and/or induction of cell death, as observed in next set of studies.

Inhibition of Cell Growth and Induction of Cell Death

In order to assess the effect of silymarin, genistein, and EGCG on DU145 cell growth and/or death, cells in the exponential growth phase were treated with 50, 100, 150, and 200 μM doses of these agents for 5 days. A dose- and time-dependent inhibitory effect of these agents was observed on cell growth. Compared to untreated control, addition of ethanol (as vehicle) did not result in an alteration in cell growth (data not shown). As shown in Figure 7A, 150 and 200 μM doses of silymarin resulted in almost complete inhibition ($P < 0.001$) of cell growth while 100 μM silymarin accounted for 70% inhibition ($P < 0.001$) after 5 days of treatment. About 50% inhibition ($P < 0.001$) was evident at 50 μM dose of silymarin following 5 days of treatment (Fig. 7A). None of the silymarin doses tested showed any cytotoxicity during 5 days of treatment (Fig. 7B). A much strong effect was observed with genistein that showed 80% inhibition ($P < 0.001$) of cell growth at 50 μM dose and complete inhibition ($P < 0.001$) at higher doses following 5 days of treatment (Fig. 7A). This marked cell growth inhibitory effect of genistein could possibly be associated with its cytotoxic effect because other than the 50 μM dose, all the other doses tested showed 30–40% cell death ($P < 0.001$) following 5 days of treatment (Fig. 7B). Similar to genistein, EGCG also showed a strong cell growth inhibitory effect causing 76% inhibition ($P < 0.001$) at 50 μM dose and complete inhibition ($P < 0.001$) at higher doses during all the time points

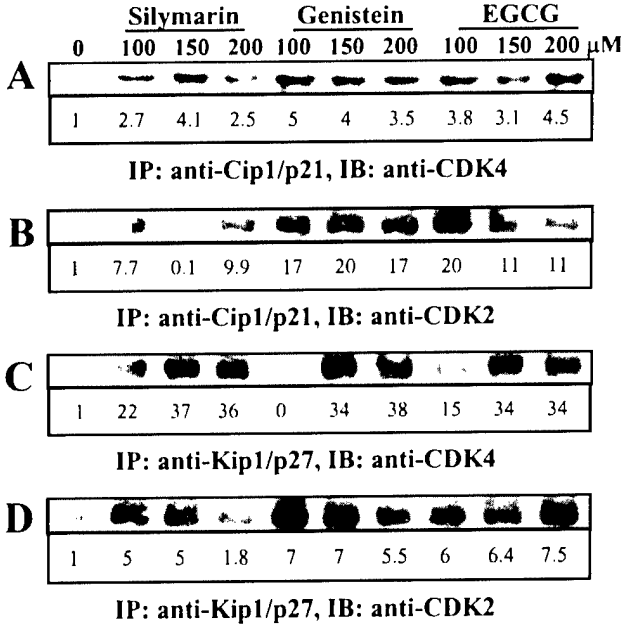

Fig. 6. Silymarin, genistein, and EGCG induce the binding of CDKIs with CDKs in DUI45 cells. Cell culture conditions, treatments, and other details are those described in Figure 4. At the end of treatments, cell lysates were prepared, Cipl/p2l and Kipl/p27 were immunoprecipitated, and subjected to SDS-PAGE followed by Western blotting as detailed in Methods. The membrane was probed with anti-CDK4 (**A**, **C**) or CDK2 (**B**, **D**) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lane I labeled as 0 denotes ethanol treatment alone. IB, Western immunoblotting. The densitometric data (arbitrary numbers), shown under the immunoblots in each panel, are average of three independent experiments with less than ± I0% variation.

studied (Fig. 7A). These growth inhibitory effects of EGCG were largely due to its high cytotoxic effects. As shown in Figure 7B (bottom panel), after 24 hr of EGCG treatment, 100, 150, and 200 μM doses resulted in 35–80% cell death ($P < 0.001$), and after 5 days of treatment, these doses of EGCG caused complete cell death ($P < 0.001$).

DISCUSSION

The central finding in the present study is that the three phytochemicals, which have been shown to be cancer-preventive and anti-carcinogenic agents against various cancers in both long- and short-term study models, showed a strong inhibitory effect against epigenetic events associated with advanced and androgen-independent human PCA growth. PCA, pertaining to its multifocal and invasive nature, and extensive mortality associated with this malignancy, attracts immense attention for its prevention, control, and therapy. Asian men have the lowest PCA incidence while, in contrast, African followed by Caucasian Americans represent the most affected population with a PCA incidence over 100-fold higher in the United States [31]. Current evidence suggests that these geographical and racial–ethnic variations in PCA incidence may, in part, be due to genetic differences as well as those in dietary habits, and androgen secretion and metabolism [31].

Epidemiological studies have shown that even with the same incidence of latent small or non-infiltrating prostatic carcinomas, the incidence of clinical PCA and associated mortality is low in Japan and some other Asian countries [32]. These data suggest a hypothesis that "although the initiation of PCA is inevitable, targeting epigenetic events could control its progression to clinical cancer, and that the incidence of clinical PCA is low in Asian countries because of their dietary habits that also include the nutrition rich in several flavonoids and isoflavones" [33]. Consistent with this hypothesis, results of the present study show that silymarin, genistein, and EGCG significantly inhibit erbB1-mediated mitogenic signaling in advanced and androgen-independent human PCA DU145 cells, suggesting the possibility that this could be one of the reasons for the observed low incidence of clinical PCA in Asian men. These results also suggest that more studies are needed with these agents to develop them for the prevention and/or intervention of PCA growth and its metastatic potential.

An erbB1-mediated mitogenic signaling pathway activates Shc-Grb2-ras-raf signaling that causes the activation of ultimate cytoplasmic target, the MAPK/ERK1/2 [3,34]. The activated MAPK/ERK1/2 then translocate to the nucleus and activate transcription factors for cell growth and proliferation [3,34]. In case of advanced and androgen-independent PCA, several studies have shown genetic alterations resulting in an enhanced expression of erbB1 and associated ligand that leads to an epigenetic mechanism of autocrine growth loop via ligand/erbB1 interaction [8–10]. Together, these studies suggest that growth factors and receptors associated with PCA progression regulate cell growth mostly through the activation of MAPKs. Indeed, recent studies have shown that MAPK/ERK1/2 are constitutively very active in DU145 cells; and that epidermal growth factor, insulin-like growth factor-1 and protein kinase A activator significantly activate MAPK/ERK1/2 in both LNCaP and DU145 human PCA cells via erbB1 receptor [35]. In addition, an increase in constitutive activation of MAPK/ERK1/2 signaling has been reported in human PCA as it progresses to a more advanced and androgen-independent malignancy [36]. Consistent with the involvement of activated MAPK/ERK1/2, possibly via TGFα/erbB1 autocrine loop, in the progression of

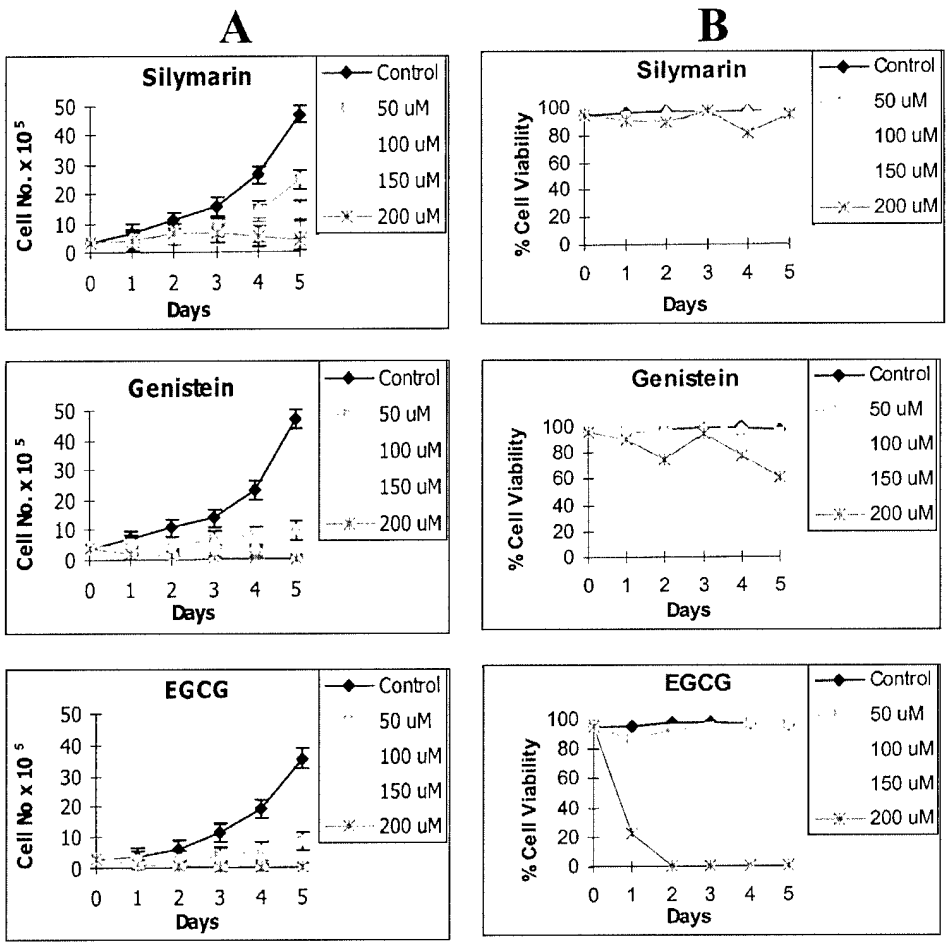

Fig. 7. Silymarin, genistein, and EGCG cause significant growth inhibition (**A**) of DUI45 cells, but only genistein and EGCG induce cell death (**B**). The details of cell culture and treatments are those described in Methods. Each data point represents mean ± SE of three independent plates, each sample was counted in duplicate.

advanced and androgen-independent human PCA, as summarized in Figure 8, we observed that impairment of erbB1-Shc activation results in the inhibition of MAPK/ERK1/2 activation in DU145 cells by silymarin and genistein. However, with EGCG treatment, an inhibition of erbB1-Shc activation did not result in the impairment of MAPK/ERK1/2 activation, but caused an additional stimulation. Studies are in progress to define the mechanism of this effect.

It has been shown that signaling pathways determine cell growth and inhibition through cell cycle regulation [10,18,37]. Using erbB1 blocking antibody, recent studies have shown that impairment of erbB1 activation leads to inhibition of Shc activation followed by selective induction of Kip1/p27 and G_1 arrest in DU145 cells [10,18]. These results further support a direct cause and effect relationship between impairment of erbB1-mediated mitogenic signaling and perturbations in cell cycle regulation leading to cell growth inhibition [10,18]. Furthermore, a growth-promoting mitogenic signal is known to be involved in normal cell proliferation via cell cycle progression where it commands the cells in G_0 restriction checkpoint to go through G_1, S, and G_2-M phases of the cell cycle [37]. However, in transformed cells, cell cycle progression could be a mitogenic signal-dependent or -independent process [10,18,37]. For example, several studies have shown a loss of functional CDKI in different human cancers and derived cell lines that leads to uncontrolled cell proliferation due to an increase in the function of CDK–cyclin complex [37]. Similarly, an overexpression of cyclin D1 and CDK4 has also been reported in several human malignancies that leads to shortening in G_1 phase causing uncontrolled cellular proliferation [38]. Consistent with these studies, it can be appreciated that targeting cell cycle regulators, i.e., induction of CDKIs and decrease in CDKs and cyclins, either dependent or independent of mitogenic signaling impairment, could be another strategy for the inhibition of epigenetic events associated with malignant cell growth. In this regard, whereas silymarin was less effective than genistein and EGCG in inhibiting erbB1-mediated signaling pathway, it showed a much stronger effect on the modulation of cell cycle regulators and their interplay (Fig. 8) in DU145 cells. The data obtained for cell growth inhibition and death suggest that, depending on their effects on epigenetic events, they could either

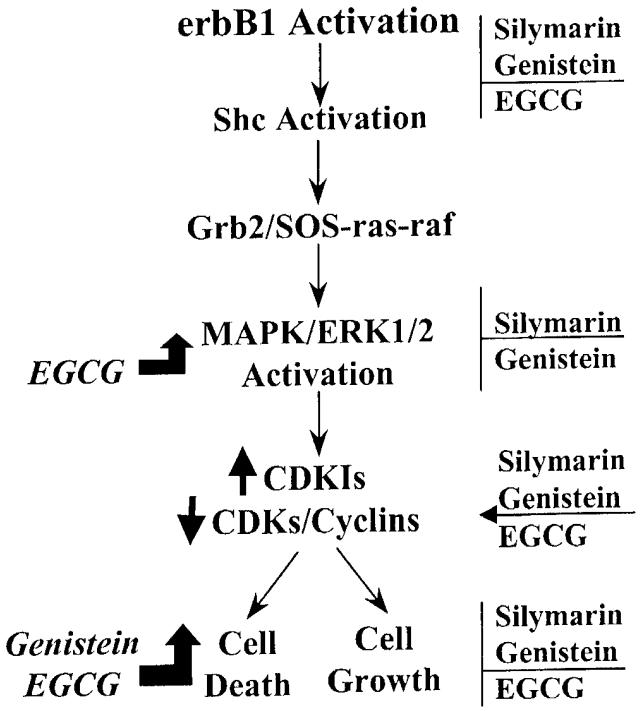

Fig. 8. A summary of the observed effects of silymarin, genistein, and EGCG on erbB1-mediated mitogenic signaling and modulation of cell cycle regulators in DU145 cells and their association with cell growth inhibition and death.

be anti-proliferative (as is the case with silymarin) or death-inducing agents (Fig. 8) against advanced and androgen-independent human prostate cancers.

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Appendix 8

MOLECULAR CARCINOGENESIS 30:1–13 (2001)

Inhibitory Effect of Silibinin on Ligand Binding to erbB1 and Associated Mitogenic Signaling, Growth, and DNA Synthesis in Advanced Human Prostate Carcinoma Cells

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We recently showed the inhibitory effect of a flavonoid antioxidant silymarin on erbB1-Shc activation in prostate cancer (PCA) DU145 cells. In the present study, we performed more detailed mechanistic and molecular modeling studies with pure compound silibinin to assess and define its effect on membrane signaling related to erbB1 activation in human PCA LNCaP and DU145 cells. Studies also were performed to establish the biologic responses toward extracellular signal-regulated protein kinase 1/2 (ERK1/2) activation, cell growth, and DNA synthesis. Treatment of serum-starved cells with various doses of silibinin for 2 h followed by ^{125}I-epidermal growth factor (EGF) showed 30–75% inhibition in ligand binding and 55–95% inhibition in its internalization in LNCaP cells, and 20–64% and 12–27% inhibition in these two events in DU145 cells. Time-response studies showed similar effects. In further studies, treatment of serum-starved cultures with silibinin followed by EGF showed strong inhibitory effects on membrane and cytoplasmic signaling molecules. In the case of erbB1 activation, silibinin showed 58–75% decrease in LNCaP and 40–100% decrease in DU145 cells at 50, 75, and 100-μg/mL doses. Inhibitory effects of silibinin also were evident on ~~erbB1 dimerization (50% to complete inhibition) and~~ ERK1/2 activation (20–80% inhibition) in both cell lines. Treatment of serum-starved cultures with silibinin resulted in 20–40% and 30–55% inhibition of LNCaP and DU145 cell growth, respectively, at similar doses after 1–3 d of treatment, and 10–50% cell death in both cell lines. Under 10% serum conditions, identical silibinin treatments resulted in 20–65% inhibition of cell growth in LNCaP and DU145 cells but did not cause any cell death. Similar doses of silibinin treatments for 24 h also resulted in 25–60%, 35–40%, and 36–50% inhibition of DNA synthesis when cells were cultured in 10% serum, totally serum starved, and serum starved plus stimulated with EGF, respectively. Molecular modeling of silibinin showed that it is a highly lipophilic compound, suggesting that it interacts with lipid-rich plasma membrane including binding with erbB1, thereby competing with the EGF–erbB1 interaction. Because the ligand-erbB1 autocrine-loop is causally involved in advanced and androgen-independent PCA, the observed effects of silibinin and its strong lipophilic nature could be useful in developing this agent for the prevention and therapy of PCA. *Mol. Carcinog. 30:1–13, 2001.*

Key words: ~~7777~~ silibinin, Receptor signaling, Prostate Cancer

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INTRODUCTION

Prostate cancer (PCA) is the most invasive and frequently diagnosed malignancy and the second leading cause (after lung) of cancer deaths in American males [1]. Therefore, new approaches are needed to control advanced androgen-dependent and -independent PCA and prevent the disease from progressing. One approach to reduce PCA incidence, growth, and metastasis is its prevention and therapy targeted toward the mitogenic and survival signaling mediated by growth factor receptors [2–8]. This approach is based on the rationale that enhanced tyrosine kinase activity due to overexpression of receptor tyrosine kinases (RTKs) and/or non-RTKs leads to persistent autocrine stimulation of cells for uncontrolled growth by secreted growth factors, which in turn can lead to disease [9].

Consistent with this notion, the aberrant expression of erbB family members of RTKs such as epidermal growth factor receptor (EGFR or erbB1), erbB2, and erbB3 was well documented, with strikingly high frequency, in prostatic intraepithe-

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Abbreviations: PCA, prostate cancer; RTKs, receptor tyrosine kinases; EGFR, epidermal growth factor receptor; TGFα, transforming growth factor α; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated protein kinase 1/2; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; BrdU, bromodeoxyuridine; LP, lipophilic potential; IGF-1, insulin-like growth factor 1; IGF-1R, insulin-like growth factor 1 receptor; PI3K, phosphatidylinositol 3-kinase; IGFBP3, insulin-like growth factor binding protein 3.

lial neoplasia and invasive PCA, both primary and metastatic [2,7,10–14]. PCA progression also was associated with a transition from a paracrine to an autocrine relationship between erbB1 and transforming growth factor α (TGFα) [10]. In primary PCA, the neoplastic cells expressed erbB1 and the surrounding stromal cells expressed TGFα, whereas in advanced disease the neoplastic cells coexpressed erbB1 and TGFα [10]. In addition, epidermal growth factor (EGF), TGFα, and erbB1 were associated with the regulation of prostatic cell mitogenesis [15]. For example, hormone-independent prostate carcinoma cells commonly express high levels of erbB1 and TGFα, thus making a functional autocrine loop for the hormone-independent growth of PCA [16,17]. With hormone-independent prostate carcinoma cell lines PC-3 and DU145, high-affinity, ligand-blocking monoclonal antibodies to erbB1 were shown to prevent its activation and result in the growth inhibition of these cells [16–18]. Together, these studies implicated the erbB family of RTK-mediated signaling pathways as contributory mechanisms for human PCA; therefore, one practical and translational approach for the prevention and therapy of PCA might be to identify the inhibitors of erbB family of RTK-mediated signaling pathway(s).

Several studies suggested that non-nutritional agents present in fresh fruits, yellow-green vegetables, and various herbs reduce human cancer incidence and mortality due to stomach, colon, breast, lung, bladder, esophageal, prostate, and other cancers [19–25]. Among these, polyphenolic antioxidants have received increased attention in recent years as cancer preventive and therapeutic agents [26–28]. Silymarin is a polyphenolic flavonoid isolated from milk thistle (*Silybum marianum* (L.) Gaertn) and is composed mainly of stereoisomer silibinin (~90%, w/w) [29]. For more than 25 y, silymarin and silibinin were used clinically in Europe as antihepatotoxic agents [30–32]. In recent years, silymarin also was used as a therapeutic agent against liver diseases in Asia and the United States and sold as a dietary supplement in the United States and Europe. Toxicity data on silymarin and silibinin as therapeutic agents showed that they are exceptionally well tolerated and largely free of adverse effects [33–36].

Recent studies from our laboratory showed strong cancer preventive and anticarcinogenic efficacies of silymarin in different epithelial carcinogenesis models such as skin cancer and in breast, skin, and cervical carcinoma cells [37–42]. With regard to PCA, we recently showed that treatment of human prostate carcinoma DU145 cells with silymarin inhibits constitutive (10% serum condition) erbB1 activation and activation caused by TGFα and then impairs downstream activation of the signaling molecule Shc. These inhibitory effects of silymarin on erbB1–Shc activation in DU145 cells also modulated cell-cycle regulators, leading to a G_1 arrest followed by inhibition of anchorage-dependent and -independent cell growth [43]. In another study, we showed that pure stereoisomer silibinin inhibits prostate-specific antigen levels in human prostate carcinoma LNCaP cells, leading to perturbations in cell-cycle regulators and cell growth inhibition followed by neuroendocrine differentiation [44].

Ligands that specifically bind and activate erbB1 include EGF and TGFα [45,46]. Activation of erbB1 by ligand binding consists of ligand–receptor internalization, receptor dimerization, activation of intrinsic receptor tyrosine kinase activity, autophosphorylation of receptor carboxyl terminus, and tyrosine phosphorylation of intracellular signaling molecules [9,45,46]. Based on our recent observations showing the inhibitory effect of silymarin on erbB1–Shc activation in DU145 cells, in the present study, we performed more detailed mechanistic studies with pure compound silibinin to assess and define its effect on membrane signaling related to erbB1 activation in human prostate carcinoma LNCaP and DU145 cells. The biologic response of silibinin on the erbB1 activation mechanism also was assessed in terms of its effect on the ultimate cytoplasmic mitogenic signaling molecule, extracellular signal-regulated protein kinase 1/2 (ERK1/2) activation, and cell growth and DNA synthesis. In addition, we performed computational three-dimensional modeling of silibinin with molecular modeling software (SYBYL version 6.5, Tripos, St. Louis, MO). With the MOLCAD feature of this software, we generated the lipophilic potential contours across silibinin so we could visualize the distribution of lipophilic portions of the molecule. This visualization led us to hypothesize that silibinin competes with the ligand–erbB1 interaction at the lipid-rich plasma membrane.

MATERIALS AND METHODS

Cells and Culture Conditions

Androgen-dependent LNCaP and androgen-independent DU145 human prostate carcinoma cells were obtained from American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 culture medium containing 10% fetal bovine serum and 1% penicillin-streptomycin antibiotics (all cell culture materials were obtained from Gibco BRL, Gaithersburg, MD) under standard culture conditions at 37°C, 95% air and 5% CO_2, and 90–95% humidity. The cultures grown and maintained under these conditions were used in all studies.

Ligand Binding to erbB1 and Ligand Internalization

First we did a dose-dependent study to assess the effect of silibinin on ligand binding to erbB1 and

ligand internalization in both cell lines. These studies were performed as described by Baulida et al. [46] with modifications. LNCaP and DU145 cells were seeded at 0.12 million cells/well in 12-well dishes under standard culture conditions, and, after 24 h, the cells were subjected to serum starvation. Briefly, the attached cells were quickly washed two times with phosphate-buffered saline (PBS) and replaced with fresh medium without serum. This serum starvation was necessary to shut down the constitutive activation of erbB1 and make the receptor available for ligand binding. After 34 h under these serum-starvation conditions, the cultures were treated with dimethylsulfoxide (DMSO) or various concentrations (50, 75, 100, and 150 μg/mL) of silibinin (obtained commercially from Sigma Chemical Co, St. Louis, MO) in DMSO. The purity of silibinin was 100% according to results obtained with high-performance liquid chromatography [47]. The final concentration of DMSO in each treatment including control was 0.5% (v/v) of the medium. The silibinin doses in the present study were the same as those in previous studies and are not cytotoxic [38–44]. Two hours after these treatments, cultures were incubated with ^{125}I-EGF (2 ng/mL, or 0.28 μCi, specific activity 900 Ci/mmol; Amersham Pharmacia Biotech, Piscataway, NJ) at 37°C for 6 min. Medium was then aspirated, and cultures were rapidly washed with ice-cold medium. The surface-bound ^{125}I-EGF was removed by a rapid wash with 0.2 mL of glacial acetic acid (pH 2.8), added to 5 mL of scintillation fluid, and quantitated as a measure of surface-bound ligand. The cells were then solubilized in 0.2 mL of 1 M NaOH, added to 5 mL of scintillation fluid, and counted to determine internalized ^{125}I-EGF (ligand). The nonspecificity of binding and internalization of ligand was determined by adding 400 ng (200-fold excess) of unlabeled EGF 5 min before hot ligand.

Based on the results from dose-dependent study (see Results), we did a time-response study to assess the effect of time of silibinin treatment on ligand binding to erbB1 and ligand internalization in both cell lines. Under identical conditions, 34-h serum-starved LNCaP and DU145 cells were treated with DMSO alone or 100 μg/mL of silibinin in DMSO for different times. Cultures were then incubated with ^{125}I-EGF (2 ng/mL) at 37°C for 6 min. Medium was aspirated, and cultures were rapidly washed with ice-cold medium. The levels of surface-bound ^{125}I-EGF and internalized ^{125}I-EGF (ligand) were then measured. In another study, serum-starved cultures were first treated with ^{125}I-EGF for 6 min followed by DMSO alone or 100 μg/mL of silibinin in DMSO for 0.5, 1, and 2 h. The levels of surface-bound ^{125}I-EGF and internalized ^{125}I-EGF (ligand) were then measured.

ErbB1 and ERK1/2 Activation

For these studies, 60% confluent LNCaP and DU145 cultures were washed twice with PBS and then starved in serum-free medium for 36 h, with one serum-free medium change after 20 h. During the last 2 h of starvation, the cultures were treated with DMSO or various doses of silibinin (50, 75, and 100 μg/mL of medium) in DMSO. At the end of these treatments, cultures were added to PBS alone or EGF (50 ng/mL of medium) and incubated for 15 min at 37°C. Medium was aspirated, cultures were quickly washed two times with cold PBS, and cell lysates were prepared as described elsewhere [43]. For erbB1 activation studies, 400 μg of protein lysate per sample was subjected to immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting, as described elsewhere [43]. Membranes were probed with antiphosphotyrosine or anti-EGFR antibody (Upstate Biotechnology, Lake Placid, NY) followed by peroxidase-conjugated appropriate secondary antibody and visualization by the enhanced chemiluminescence detection system [43]. For ERK1/2 acti vation studies, 50 μg of protein lysate per sample was denatured with 2 × sample buffer, samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gel, and separated proteins were transferred onto membranes by western blotting. Membranes were probed with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies (New England Biolabs, Beverly, MA) followed by the desired secondary antibodies and detection by the enhanced chemiluminescence system.

In each case, western immunoblots were scanned with Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed with Scanimage (National Institutes of Health, Bethesda, MD). For each case, only representative blots are shown with reproducible findings in three independent experiments. However, the densitometric data shown in each case are the mean of three independent experiments with less than 10% variation.

Cell Growth and DNA Synthesis

The inhibitory effect of silibinin on cell growth was assessed under serum-starved and serum-growth conditions. Cells were plated at a density of 1×10^5 cells/60-mm plate. After 24 h, cells were serum starved for 36 h and then treated with DMSO or 50, 75, and 100 μg/mL of silibinin in DMSO. The cultures were fed with fresh medium without serum and the same concentrations of silibinin after 48 h (in a 72-h study). After 1–3 d of treatments, cells were trypsinized and cell number was determined with a hemocytometer. In another study, cells were

plated at a density of 1×10^5 cells/60-mm plate in complete medium (with 10% serum). After 24 h, cells were added to fresh medium containing 10% serum and treated. After 1–3 d, cells were trypsinized and cell number was determined.

The effect of silibinin on DNA synthesis was assessed by incorporating bromodeoxyuridine (BrdU) with a colorimetric enzyme-linked immunosorbent assay kit (Boehringer Mannheim, Indianapolis, IN). Several different treatment protocols assessed the effect of silibinin on DNA synthesis with serum and serum-starved conditions with or without EGF after silibinin pretreatment. Briefly, in the first study, 1000 cells/well were cultured in 96-well plates at 37°C for 24 h in medium containing 10% serum and then refed with fresh medium containing DMSO or 50, 75, and 100 μg/mL of silibinin in DMSO. After 24 h, BrdU was added and the medium was incubated for another 2–3 h at 37°C. Thereafter, DNA was denatured and cells were incubated with anti-BrdU antibody followed by addition of substrate. The reaction product was quantified by measuring absorbance at a 450-nm wavelength with a scanning multiwell spectrophotometer. In other studies, 24 h after identical cell seeding, cells were washed with serum-free medium and serum starved for 34 h. Those cells were then treated with the same doses of silibinin alone or silibinin followed 2 h later by the addition of EGF (50 ng/mL). After 24 h, DNA synthesis was determined for both treatments.

Statistical Analysis

The data in each case were analyzed with one-way analysis of variance. If this analysis indicated at least one significant difference (with $P = 0.05$ as the cut-off value), pairwise comparisons were performed. The type I error rate was kept at 0.05 with the Bonferroni correction for multiple comparisons. For three comparisons, if a difference is to be regarded as statistically significant, the Bonferroni correction requires $P < 0.0167$ for any given pairwise comparison. Pairs of treatment groups that were statistically significant (vs. DMSO control; see Results) are based on these post hoc tests.

Molecular Modeling of Silibinin

Molecular modeling was done on a Silicon Graphics Indigo2 Impact 10000 workstation using the molecular modeling program SYBYL. The molecule was energy minimized for geometry and electrostatic interactions by using semiempirical molecular orbital methods with the AM1 algorithm [48,49]. The fully protonated molecule and a dielectric constant of one was used. The program MOLCAD (molecular computer aided design) [50], which is distributed by Tripos as an optional SYBYL module, was used to generate the lipophilic potentials (LPs) of silibinin. First, the electron-density surface was created with the charges calculated by the AM1 algorithm. The density function generated an isosurface that qualitatively represented the electron density. The LP property was then calculated [51–53] and mapped on the existing electron-density surface. The color ramp for LP ranges from brown (highest lipophilic area of the molecule) to blue (highest hydrophilic area).

RESULTS

Effect of Silibinin on Ligand Binding to erbB1 and Ligand Internalization

Silibinin treatment of LNCaP (Figure 1A) and DU145 (Figure 1C) cells resulted in strong inhibition (in a dose-dependent manner) of ligand binding to erbB1 and internalization of the ligand. When the results were analyzed for LNCaP cells (Figure 1A), silibinin treatment at the 50-μg/mL dose showed little inhibitory effect toward ligand binding to erbB1; however, the higher doses of 75, 100, and 150 μg/mL of silibinin resulted in statistically significant (vs. DMSO control) inhibition (30%, 50%, and 75%, respectively). With regard to ligand internalization in LNCaP cells, these four doses of silibinin resulted in much stronger effects, accounting for 55%, 70%, 83%, and 95% inhibition (statistically significant at all dose levels vs. DMSO) at doses of 50, 75, 100, and 150 μg/mL, respectively. In the case of DU145 cells (Figure 1C), a reverse trend was observed toward the inhibitory effect of silibinin on ligand binding to erbB1 and its internalization. Whereas 50 μg/mL of silibinin showed only 20% inhibition, statistically significant (vs. DMSO) inhibition was evident at 75, 100, and 150 g/mL of silibinin, accounting for 35%, 50%, and 64% inhibition in ligand binding to erbB1 (Figure 1C). For ligand internalization, these doses of silibinin showed no (at 50 μg/mL), weak (at 75 and 100 μg/mL), or moderate but statistically significant (at 150 μg/mL) inhibition. An analysis of the results for nonspecific binding with 200-fold excess of cold ligand showed that it was within 5% of specific binding for hot ligand (data not shown).

In the studies assessing the time-dependent pretreatment effect of silibinin, silibinin treatment (at 100 μg/mL) of LNCaP (Figure 1B) and DU145 (Figure 1D) cells resulted in strong inhibition, in a time-dependent manner, of ligand binding to erbB1 and ligand internalization. When results were analyzed for LNCaP cells (Figure 1B), silibinin treatment for 10 min showed a weak inhibitory effect (15%) toward ligand binding to erbB1, whereas longer treatment times (0.5–24 h) resulted in statistically significant (vs. DMSO) inhibition, accounting for 40–100% inhibition. In terms of ligand internalization in LNCaP cells, all the time points examined showed statistically significant inhibition (30–100%) as soon as 10 min to 24 h after silibinin treatment. In the case of DU145 cells (Figure 1D),

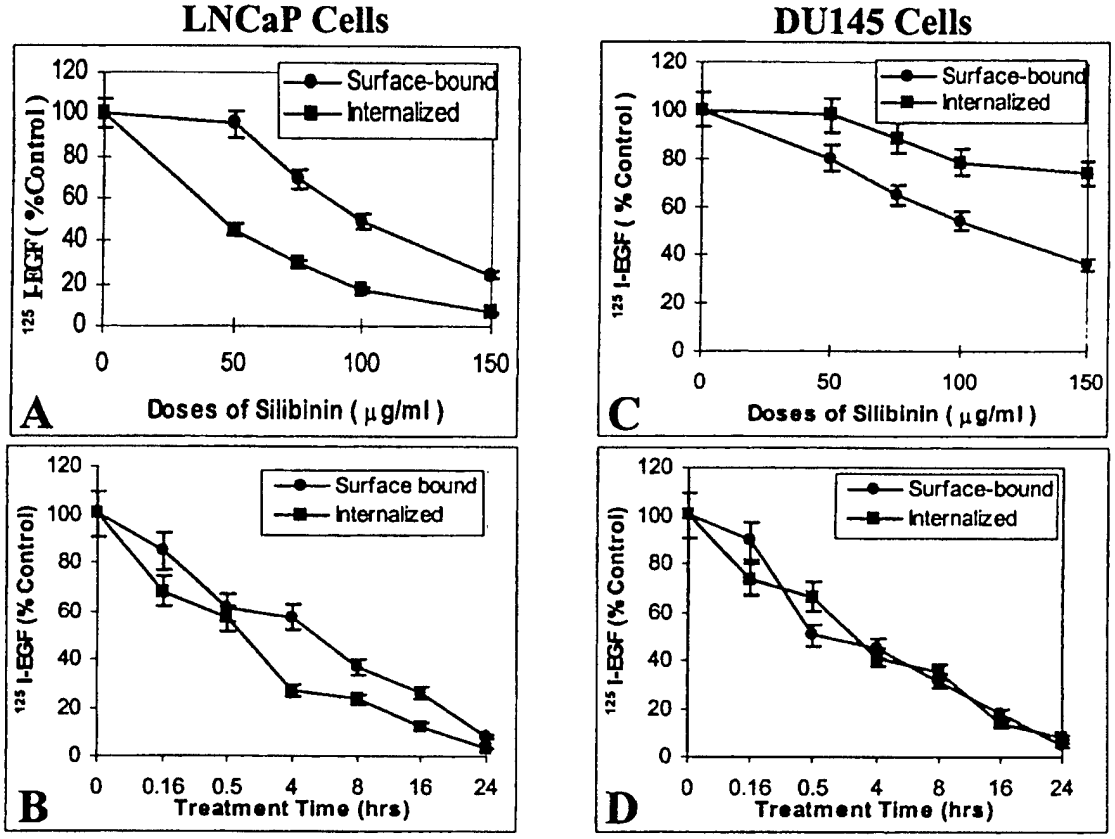

Figure 1. Effect of silibinin on ligand binding and internalization. In a dose-response study, LNCaP (A) and DU145 (C) cells were seeded at 0.12 million cells/well in 12-well dishes and after 24 h were serum starved for 34 h. The cultures were then treated with DMSO or various doses (50, 75, 100, and 150 μg/mL) of silibinin for 2 h followed by ^{125}I-EGF at 37°C for 6 min. The surface-bound and internalized ^{125}I-EGF levels were analyzed as detailed in Materials and Methods. In a time-response study, starved LNCaP (B) and DU145 (D) cultures were treated with 100 μg/mL dose of silibinin for the indicated times followed by ^{125}I-EGF, and ligand binding to erbB1 and ligand internalization were determined as detailed in Materials and Methods. In each case, the data shown are mean ± SD of two independent studies; each study had three independent plates.

similar time-dependent inhibitory effects of silibinin on ligand binding to erbB1 and its internalization were evident, accounting for 1[illegible]-100% inhibition of ligand binding to erbB1 and 1[illegible]-100% inhibition of ligand internalization.

In other studies, we treated the starved cells with EGF for 6 min, added silibinin at different times, and then assessed ligand binding to erbB1 and ligand internalization. In the case of LNCaP cells (Figure 2A), silibinin treatment (at 100 μg/mL) for 0.5 h resulted in low inhibition of ligand binding to erbB1 and ligand internalization. However, longer treatment times showed strong inhibition (35% and 60% at 1 and 2 h, respectively) of ligand binding and considerable inhibition (25% and 30%) of ligand internalization (statistically significant in both cases vs. DMSO; Figure 2A). In the case of DU145 cells (Figure 2B), comparable inhibitory efficacies of silibinin on ligand binding and internalization were observed after hot ligand treatment and accounted for 8–12%, 14–18%, and 40–50% inhibition (statistically significant vs. DMSO control) after 0.5, 1, and 2 h, respectively, of ligand treatment.

Effect of Silibinin on Internalized/Surface-bound Ligand Ratio

The binding of the ligand to its receptor rapidly induces internalization of the ligand–receptor complex by endocytosis, which involves clustering of this complex in coated pits followed by lysosomal degradation of the ligand–receptor complex [45, 46]. Based on data showing that silibinin inhibited ligand binding to erbB1 and ligand internalization in dose- and time-dependent manners, we assessed the relationship between internalized and surface-bound ligand in LNCaP and DU145 cells. In vehicle-treated controls, the ratio of internalized to surface-bound ligand was approximately 4.5-fold higher in LNCaP cells (Figure 3A) than in DU145 cells (Figure 3B), suggesting that EGF is internalized rapidly in LNCaP cells but not in DU145 cells. Silibinin treatment rapidly changed this trend. In the case of LNCaP cells, a statistically significant decrease (~50%) in this ratio was observed with a dose as low as 50 μg/mL (Figure 3A). The highest dose of silibinin, 150 μg/mL, decreased this ratio by up to

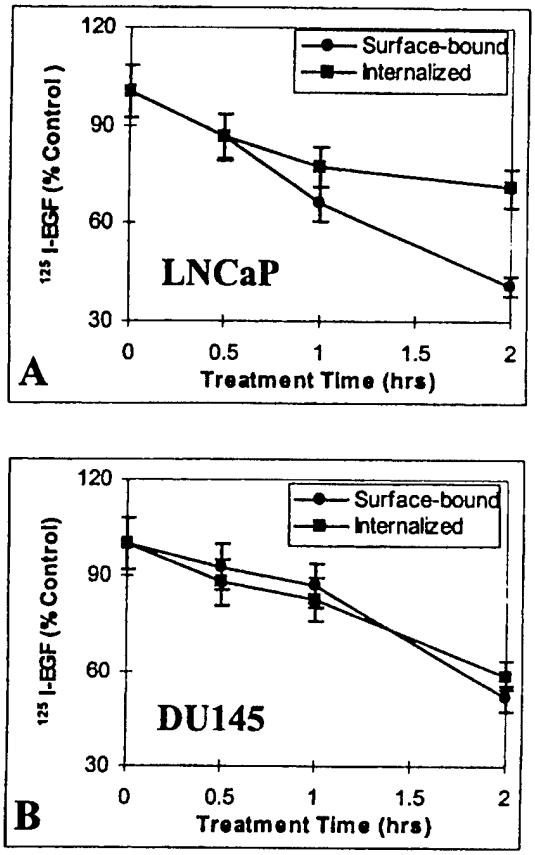

Figure 2. Effect of silibinin on ligand binding and internalization when treated after EGF. LNCaP (A) and DU145 (B) cells were seeded at 0.12 million cells/well in 12-well dishes and after 24 h were serum starved for 36 h. The cultures were then treated with ^{125}I-EGF at 37°C for 6 min followed by a 100-μg/mL dose of silibinin for the indicated times. The ligand binding to erbB1 and ligand internalization were determined as detailed in Materials and Methods. In each case, the data shown are mean ± SD of two independent studies; each study had two independent plates.

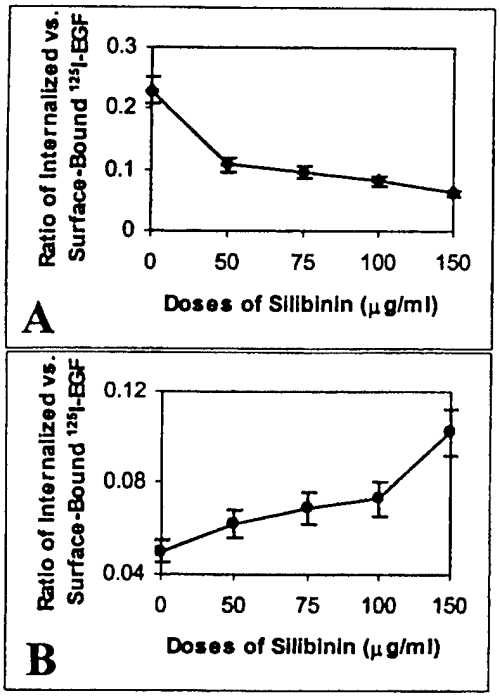

Figure 3. Effect of silibinin on internalized versus surface-bound ligand ratio. LNCaP (A) and DU145 (B) cells were seeded at 0.12 million cells/well in 12-well dishes and after 24 h were serum starved for 34 h. The cultures were then treated with DMSO or various doses (50, 75, 100, and 150 μg/mL) of silibinin for 2 h followed by ^{125}I-EGF at 37°C for 6 min. The surface-bound and internalized ^{125}I-EGF levels were used to determine the ratio of internalized versus surface-bound ligand. This study, although confirmatory to that shown in Figure 1, was done independently. In each case, the data shown are mean ± SD of four independent experimental plates from one study.

70% in LNCaP cells (Figure 3A). For DU145 cells, similar silibinin treatments resulted in dose-dependent increases in the ratio of internalized to surface-bound ligand, and the highest dose used showed a statistically significant increase (approximately two-fold vs. DMSO) in this ratio (Figure 3B). These results were consistent with the observed effects of silibinin shown in Figure 1 and suggest that ligand turnover is enhanced in DU145 cells by silibinin, a process that leads to degradation of the ligand–receptor complex.

Effect of Silibinin on erbB1 Activation

In a recent study, we showed that treatment of DU145 cells with silymarin results in a strong inhibition of ligand-induced and constitutive activation of erbB1 [43]. In the present study, we used LNCaP and DU145 human PCA cells to investigate the inhibitory effect of the major silymarin stereoisomer silibinin on ligand binding to erbB1 and ligand internalization with ligand-induced erbB1 activation. We also investigated the effect of silibinin on erbB1 signaling in LNCaP and DU145 cells. Treatment of serum-starved LNCaP and DU145 cells with silibinin for 2 h followed by EGF resulted in a strong inhibition of erbB1 activation. In the case of LNCaP cells (Figure 4A, upper panel) compared with serum-starved cultures showing complete diminution of erbB1 activation, treatment of starved cells with 50 ng/mL of EGF resulted in a strong activation of erbB1. Pretreatment of serum-starved cells with 50, 75, and 100 μg/mL of silibinin for 2 h followed by similar EGF treatment resulted in a strong inhibition of ligand-caused erbB1 activation. Densitometric analysis of blots from three independent studies showed that the observed inhibition was statistically significant and accounted for 58%, 73%, and 75% decreases in phosphorylated erbB1 at 50, 75, and 100 μg/mL of silibinin, respectively, compared with the blot treated with EGF alone (Figure 4A, upper panel). In the case of DU145 cells, silibinin pretreatment also resulted in a statistically significant inhibition of EGF-induced erbB1 activation (Figure 4B, upper panel). In this case, however, the effect was more dose dependent: doses of 50, 75, and 100 μg/mL showed 40%, 65%, and 100% inhibition, respectively, of EGF-induced

erbB1 activation (Figure 4B, upper panel). In LNCaP and DU145 cells, the observed inhibition in erbB1 activation was not caused by a decrease in erbB1 protein levels (Figure 4A and B, lower panels), suggesting a direct association of inhibition of ligand binding with the observed inhibition in erbB1 activation by silibinin.

Effect of Silibinin on ERK1/2 Activation

Constitutive activation of ~~microtubule-associated~~ mitogen-activated protein kinase/ERK1/2, possibly due to the ligand–erbB1 autocrine growth loop, was well documented in advanced and androgen-independent human prostate carcinoma cells and human PCA [54,55]. Based on the results discussed in the previous section, we investigated the effect of silibinin on ligand-induced ERK1/2 activation to (i) associate those results with the ultimate cytoplasmic mitogenic signaling target ERK1/2 in a classic erbB1 signaling cascade and (ii) establish the biologic response of the observed effect of silibinin on membrane signaling. Consistent with its inhibitory effect on erbB1 membrane signaling (Figure 5), treatment of LNCaP and DU145 cells with silibinin resulted in a statistically significant inhibition of EGF-induced ERK1/2 activation. In the case of LNCaP cells, densitometric analysis of blots from three independent studies (Figure 5A, upper panel) showed that pretreatment with 50, 75, and 100 μg/mL of silibinin for 2 h followed by EGF stimulation results in 50%, 38%, and 45% decreases in phospho-ERK1 levels and 30%, 40%, and 30% decreases in phospho-ERK2 levels, respectively. In the case of DU145 cells, however, similar silibinin treatments resulted in 50%, 60%, and 80% decreases in phospho-ERK1 levels and 20%, 50%, and 45% decreases in phospho-ERK2 levels, respectively, compared with samples treated with EGF alone (Figure 5B, upper panel). Similar to the inhibition of erbB1 activation, the observed decreases in phospho-ERK1/2 levels in LNCaP and DU145 cells were not due to decreases in ERK1/2 protein levels (Figures 5A and 4B, lower panels), suggesting a direct association of inhibition of ligand binding with the inhibition of erbB1 activation followed by a decrease in phospho-ERK1/2 by silibinin. We previously showed the inhibitory effect of silymarin on erbB1 and Shc activation in DU145 cells [43], but this is the first study showing the inhibitory effect of silibinin on ERK1/2 activation in LNCaP and DU145 cells.

Effect of Silibinin on Cell Growth, Death, and DNA Synthesis

Based the data presented in the previous section, we investigated their relationship with the biologic effects of silibinin on cell growth and DNA synthesis. We investigated the effect of silibinin on the growth of 36-h serum-starved LNCaP and DU145 cells. Serum starvation of cells for 36 h and their

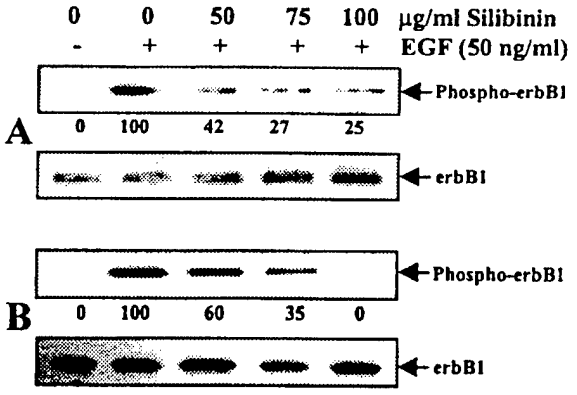

Figure 4. Effect of silibinin on erbB1 activation. LNCaP (A) and DU145 (B) cells at 60% confluency were serum starved for 36 h and during the last 2 h of starvation were treated with DMSO or various doses of silibinin in DMSO. Cultures were then treated with PBS alone or EGF (50 ng/mL of medium) for 15 min at 37°C. Cell lysates were prepared, and erbB1 was immunoprecipitated followed by SDS-PAGE and western blotting, as detailed in Materials and Methods. Membranes were probed with antiphosphotyrosine (upper panels in A and B) or anti-EGFR (lower panels in A and B) antibody followed by peroxidase-conjugated appropriate secondary antibody and visualization by ECL detection system. The treatment in each lane is as marked in the figure. In each case, only representative blots from three independent studies with reproducible findings are shown. The densitometric analysis data shown under the upper panels in A and B are the mean of three independent experiments with less than 10% variation.

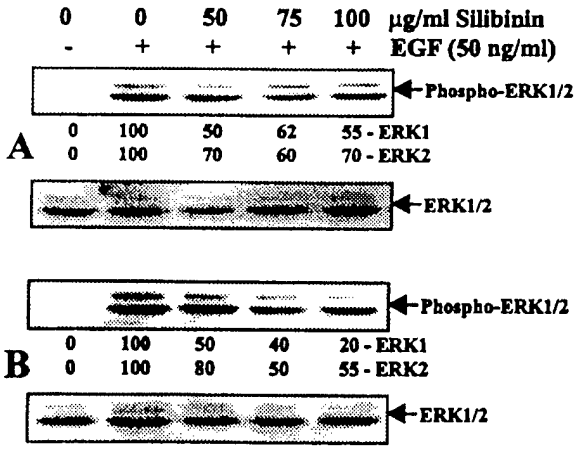

Figure 5. Effect of silibinin on ERK1/2 activation. LNCaP (A) and DU145 (B) cells at 60% confluency were serum starved for 36 h and during the last 2 h of starvation were treated with DMSO or various doses of silibinin in DMSO. Cultures were then treated with PBS alone or EGF (50 ng/mL of medium) for 15 min at 37°C. Cell lysates were prepared, and 50 μg of protein lysate per sample was subjected to SDS-PAGE and western blotting as detailed in Materials and Methods. Membranes were probed with antiphospho-ERK1/2 (upper panels in A and B) or anti-ERK1/2 (lower panels in A and B) antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by the ECL detection system. The treatment in each lane is as marked in the figure. In each case, only representative blots from three independent studies with reproducible findings are shown. The densitometric analysis data shown under upper panels in A and B are the mean of three independent experiments with less than 10% variation.

subsequent growth (without serum in the medium) for the next 3 d showed a growth pattern for 24 h followed by minimum growth up to 48 h in LNCaP cells and 72 h in DU145 cells (Figure 6A and C). Treatment of 36-h serum-starved cultures with silibinin resulted in dose- and time-dependent inhibitions of cell growth (Figure 6A and C). In LNCaP cells, 50 μg/mL of silibinin showed weak inhibition but 75 and 100 μg/mL resulted in statistically significant inhibition (~33%) of cell growth after 1 d of treatment (Figure 6A). Cell growth inhibition was again evident with 75 and 100 μg/mL after 2 d of treatment, accounting for 39% and 42% decreases, respectively (Figure 6A). Whereas overall cell growth inhibition at these doses of silibinin was less than 50% of controls, cells almost completely stopped growing as soon as after 1 d of treatment because no substantial increase in cell number was evident from that time to the end of the study (Figure 6A). In DU145 cells, these doses showed statistically significant inhibition (29–37%) after 1 d of treatment (Figure 6C). Similar to the data in LNCaP cells, silibinin treatment at all three doses caused cell growth to stop completely after 1 d of treatment (Figure 6C). When the cell growth inhibitory effects of silibinin were assessed under normal growth medium conditions (with 10% serum), similar growth inhibitory responses (statistically significant compared with control) were observed (Figure 6B and D). In LNCaP cells, these doses showed 21–31%, 37–49%, and 50–62% inhibition after 1, 2, and 3 d of treatment, respectively (Figure 6B). In DU145 cells, comparable cell growth inhibitory effects were evident (Figure 6D). In fact, all the doses of silibinin tested completely stopped the growth of LNCaP and DU145 cells (Figure 6B and D) at all time points studied.

Similar to its inhibitory effects on cell growth, silibinin treatment resulted in moderate to strong inhibition of DNA synthesis under different culture conditions. Treatment of LNCaP (Figure 7A) and DU145 (Figure 7B) cells grown under normal serum conditions with silibinin resulted in statistically significant inhibition of DNA synthesis with 50, 75, and 100 μg/mL, accounting for 35%, 42%, and 60% inhibition in LNCaP cells and 26%, 37%, and 48% inhibition in DU145 cells, respectively. When similar silibinin treatments were done in serum-starved cultures, the statistically significant inhibition was evident in both cell lines at 50 μg/mL; no further inhibition was evident by increasing the dose (Figure 7A and B). Comparable to its effect on DNA synthesis in serum-starved cells, 50 μg/mL of silibinin showed an approximately 40% inhibition

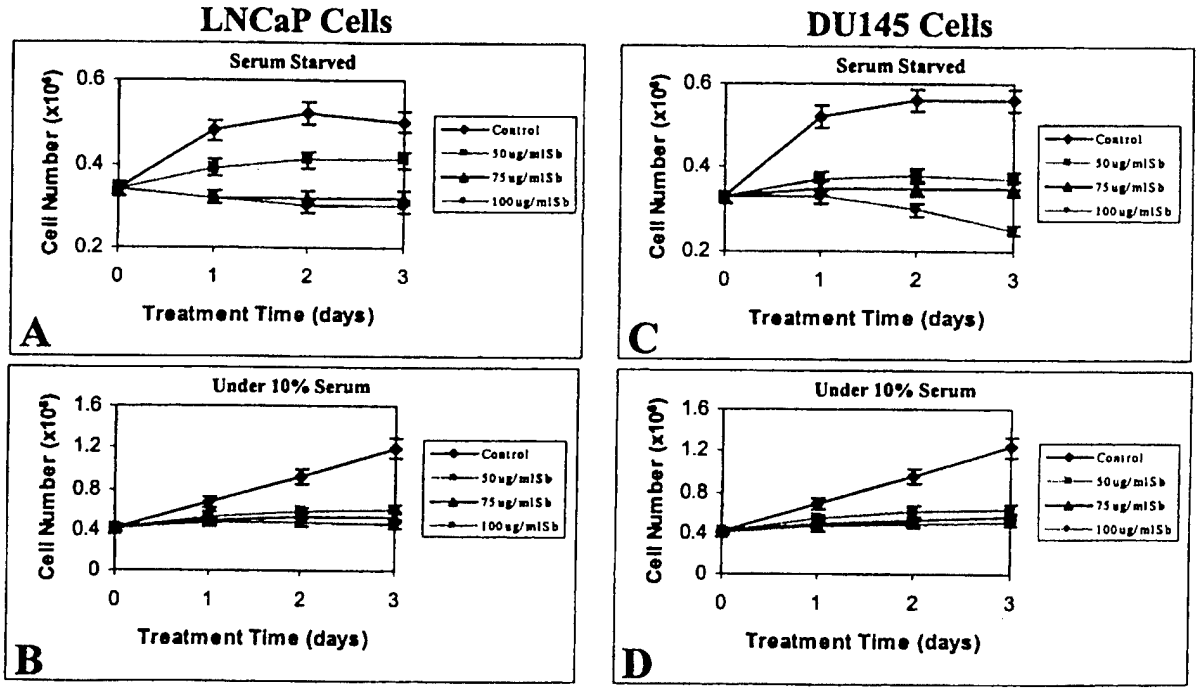

Figure 6. Effect of silibinin on cell growth. In the studies assessing the effect on serum-starved cultures, LNCaP (A) and DU145 (C) cells were plated at a density of 1×10^5 cells/60-mm plate. After 24 h, cells were serum starved for 36 h and then treated with DMSO or 50, 75, and 100 μg/mL of silibinin in DMSO. The cultures were fed with fresh medium without serum and the same concentration of silibinin after 48 h (in a 72-h study). After 1–3 d of treatment, cells were trypsinized and cell number was determined with a hemocytometer. In another study, LNCaP (B) and DU145 (D) cells were plated at a density of 1×10^5 cells/60-mm plate. After 24 h, cells were added with fresh medium with 10% serum and treated. After 1–3 d, cells were trypsinized, and cell number and viability were determined. In each case, the data shown are mean ± SD of three independent studies; each study had two independent experimental plates and each sample was counted in duplicate.

when starved cultures after 2 h of silibinin treatment were stimulated with EGF (Figure 7A and B). Higher doses in this treatment protocol showed greater inhibition of approximately 50%.

Computational Three-dimensional Modeling of Silibinin

The hydrophobic effect plays an important role in protein–protein and ligand–receptor interactions in an aqueous environment. Therefore, we also calculated the overall hydrophobicity, i.e., lipophilicity of the silibinin molecule, and the LP maps as displayed by SYBYL (Figure 8). After energy minimizing the silibinin molecule for geometry and electrostatic interactions (three-dimensional structure represented in Figure 8A and B), the LPs were generated and displayed with MOLCAD [50] (Figure 8C and D). The brown region, which represents the lipophilic portions of the molecule, suggests that silibinin is a very lipophilic molecule.

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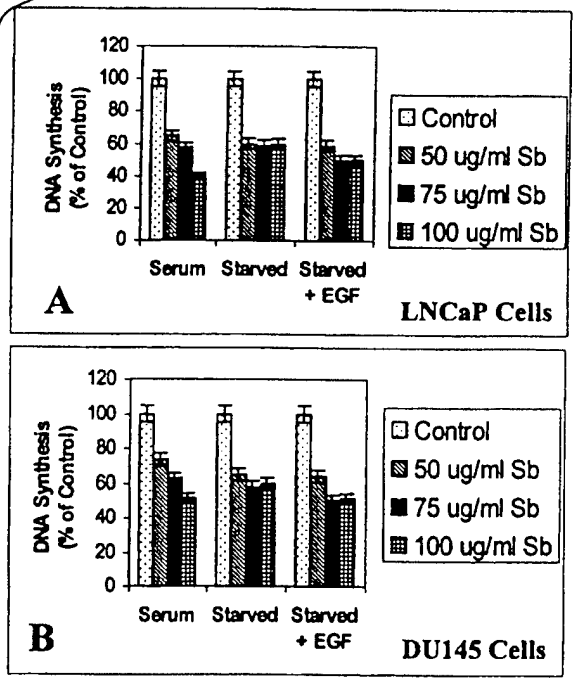

Figure 7. Effect of silibinin on DNA synthesis. LNCaP (A) and DU145 (B) cells at 1000 cells/well were cultured in 96-well plates for 24 h in medium containing 10% serum and then refed with fresh medium containing DMSO or 50, 75, and 100 μg/mL of silibinin. After 24 h, BrdU was added and then incubated for another 2–3 h. DNA was denatured and cells were incubated with anti-BrdU antibody followed by the addition of substrate. The reaction product was quantified by measuring absorbance at a 450-nm wavelength with an enzyme-linked immunosorbent assay reader, as detailed in Materials and Methods. In other studies, 24 h after identical cell seeding, cells were washed with serum-free medium and serum starved for 34 h. They were then treated with the same doses of silibinin or silibinin followed 2 h later with EGF treatment (50 ng/mL). After 24 h, DNA synthesis was determined in both sets of treatments, as detailed in Materials and Methods. In each case, the data shown are mean ± SD of two independent studies; each study used four independent experimental wells.

DISCUSSION

The central finding in the present study is that a naturally occurring flavonoid, silibinin, that is used clinically as an antihepatotoxic agent and sold as a dietary supplement, inhibits ligand binding to erbB1 receptor, ligand internalization and erbB1 activation, and ultimately cytoplasmic mitogenic target ERK1/2 activation in advanced androgen-dependent and -independent human PCA cells. Those effects of silibinin also were associated in part with its strong effect on the inhibition of cell growth and DNA synthesis. The importance of these results was emphasized by the studies showing enhanced expression and the interaction of ligand and erbB1 in advanced and androgen-independent PCA progression and metastasis potential [16–18, 43]. Consistent with this autocrine growth factor–receptor interaction, a recent study showed the constitutive activation of ERK1/2 in prostate carcinoma DU145 cells [54]. Further, another study showed that ERK1/2 is constitutively active in human PCA, and it was implicated in the progression of this malignancy in advanced and androgen-independent disease states [55]. Accordingly, the present results showing a strong decrease in phospho-ERK1/2 levels by silibinin might have strong implications for controlling PCA growth and progression to advanced and androgen-independent stages.

The ligand EGF specifically binds to the erbB1 receptor on the cellular membrane, and then this ligand–receptor complex becomes rapidly internalized by endocytosis, which involves the clustering of this complex with adaptor protein 2 in coated pits [56–59]. Several of our studies showed that silymarin and its major stereoisomer silibinin inhibit ligand-induced and constitutive activation of erbB1 in human PCA DU145 and human epidermoid carcinoma A431 cells [43,60]. In defining the mechanism of this effect at the membrane receptor level, in the present study, we found that silibinin interacts (or possibly binds) with erbB1 at the membrane and thereby inhibits EGF binding to erbB1, leading to inhibition of erbB1 activation and activations downstream of this signaling cascade such as Shc [43] and a decrease in phospho-ERK1/2 levels (present study).

As shown by the LP maps on MOLCAD surfaces (Figure 8), we found that silibinin is a highly lipophilic compound. Therefore, there are several modes of action by which silibinin can exert its biologic effects at the lipid-rich plasma membrane such as interacting with erbB1 (present study) or being translocated inside the cell to produce diversified effects [37–45]. With regard to its interaction with the plasma membrane, the lipophilic silibinin might bind directly to the membrane-associated erbB1 and thus prevent EGF from binding to its

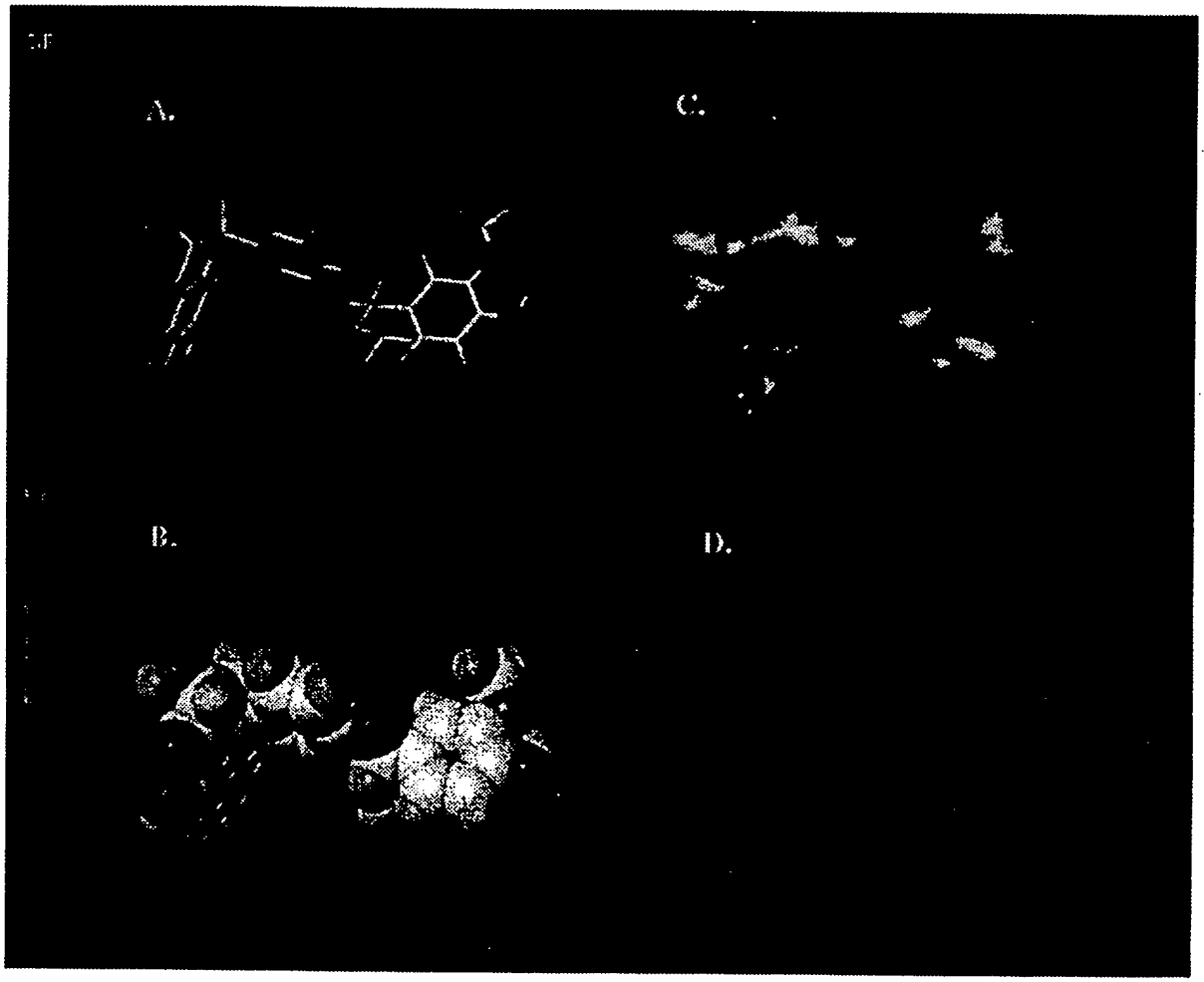

Figure 8. Computational three-dimensional modeling of silibinin. (A) Capped stick. (B) Van der Waals space filled the representation of silibinin. (C) Opaque style MOLCAD surfaces in Gouraud shading. (D) Isocontour two-dimensioanl texture mapping representing the lipophilic potential maps of silibinin. The color ramp on the left shows the LP ranges from brown (highest lipophilic area of the molecule) to blue (highest hydrophilic area).

receptor. Accordingly, it is logical to hypothesize that silibinin competes with the EGF–erbB1 interaction at the lipid-rich plasma membrane, which is responsible for the effects observed in the present study. We are in the process of starting studies with radiolabeled silibinin to address this issue further. With regard to its translocation inside the cells, silibinin itself might cross the lipid-rich plasma membrane, or it might bind to erbB1 and then become internalized by endocytosis. Although we cannot rule out the direct crossing of highly lipophilic compounds such as silibinin, studies have shown that the latter possibility is more likely [61,62]. More studies are needed to resolve these issues.

The results obtained for the inhibition of cell growth and DNA synthesis by silibinin in LNCaP and DU145 cells under normal serum conditions are consistent with those reported previously [43,44, 63]. With regard to the inhibitory effect of silibinin on cell growth and DNA synthesis in serum-starved cultures, a situation where autocrine TGFα/erbB1-mediated mitogenic signaling and associated cell growth decrease because of long-term serum starvation, as shown in the present study and elsewhere [43,64], it seems likely that silibinin produces growth inhibitory effects in addition to the inhibition of RTK-mediated mitogenic signaling. Consistent with this notion, we showed that inhibition of the erbB1-mediated signaling by silymarin in DU145 cells induces Kip1/p27 and associated events, causing G_1 arrest and growth inhibition [43]. However, we also found that a mechanism independent of the inhibition of RTK-mediated mitogenic signaling is responsible for silymarin induction of Cip1/p21 as an additional growth inhibitory response in DU145 cells [43]. Moreover, we and others have shown that, although anti-EGFR antibody clone 255 (which blocks receptor activation) strongly inhibits the growth of DU145 cells, inhibition is not complete

even at high concentrations compared with silymarin [18,43]. These findings clearly suggested that additional growth stimulating pathways, independent of erbB1-mediated mitogenic signaling, are operational in advanced and androgen-independent human PCA and derived cell lines and that silymarin also exerts inhibitory effects on them [18,43].

The present results also lead to other questions about the effect of silibinin on other RTK-mediated mitogenic and cell survival signaling pathways. For example, studies are needed to investigate whether the observed effects of silibinin in inhibiting EGF binding to erbB1 are specific or could be extended to other growth factor receptor–ligand interactions such as insulin-like growth factor 1 (IGF-1) with IGF-1 receptor (IGF-1R) and other members of the erbB–RTK family. We found that silibinin inhibits erbB1 homodimerization (unpublished observations), and future studies should investigate whether it inhibits erbB1 heterodimerization with other erbB family members or other RTKs such as IGF-1R. These studies are specifically important because constitutive activation of the phosphatidylinositol-3 kinase (PI3K)–AKT-mediated cell survival pathway was found in human PCA cells [65,66]. After IGF-1 binding, activation of IGF-1R, which activates the PI3K–AKT pathway, might be activated in part by erbB1–IGF-1R or erbB1–erbB3 heterodimerization [67,68]. A strong decrease in serum IGF binding protein 3 (IGFBP3) was reported in PCA patients, accounting for increased IGF-1–IGF-1R interaction and its association with uncontrolled proliferation of human PCA [69,70]. In addition, a nonfunctional PTEN was associated with constitutive activation of the PI3K–AKT pathway in human PCA LNCaP cells [66]. In a recent study, we showed that silibinin treatment of advanced and androgen-independent human PCA PC3 cells results in a very strong induction of IGFBP3 mRNA and protein expression [71]. In that study, we also found that silibinin strongly inhibits IGF-1–caused insulin receptor substrate 1 activation, an important molecule in IGF-1R–mediated downstream signaling [67,71]. By using IGFBP3 antisense, we also demonstrated that the inhibitory effects of silibinin on IGF-1–mediated signaling and proliferation are strongly reversed [71]. Together these data clearly demonstrate the inhibitory effect of silibinin on the IGF-1R–mediated signaling pathway via IGFBP3 upregulation. Whether silibinin directly interferes with the interaction between IGF1 and IGF-1R, as observed in the present study for EGF and erbB1, remains to be established. Studies are also needed to answer the question of whether the doses of silibinin used in the present study were physiologic and could be achieved in vivo in PCA animal models and human clinical trials. Studies are underway to establish the physiologic and pharmacologic doses of silibinin in animal models.

ACKNOWLEDGMENT

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Appendix 9

Silibinin Causes Hypophosphorylation of Rb/p107 and Rb2/p130 via Modulation of Cell Cycle Regulators in Human Prostate Carcinoma DU145 Cells

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Abstract

Phosphorylation status of retinoblastoma (Rb) and related proteins is important to drive cell cycle progression for growth. In their hyperphosphorylated states, they are growth stimulatory, but their hypophosphorylation is growth inhibitory. In several recent studies, we have shown both cancer preventive and anti-carcinogenic efficacy of silymarin and silibinin in different epithelial cancer models. Here we assessed whether silibinin causes hypophosphorylation of Rb-related proteins as its growth inhibitory response in human prostate cancer (PCA) cells. Silibinin treatment of human PCA DU145 cells resulted in a strong increase (2.3-fold) in the levels of hypophosphorylated Rb/p107 and Rb2/p130. Based on these results, next we assessed whether this effect of silibinin is via modulation of cell cycle regulators? In these studies, silibinin-treated cells showed a dose-dependent increase in Cip1/p21 and Kip1/p27 levels. Maximum induction was 13- and 6-fold in case of Cip1/p21 and Kip1/p27 following 24 and 12 hrs of treatments, respectively. Silibinin treatment also resulted in 90 and 70% decrease in CDK4 and CDK2 levels, respectively, but did not alter the protein levels of cyclin D1, cyclin E, and E2F family of transcription factors. Consistent with its effect on G1 cell cycle regulators, silibinin treated cells exhibited a strong G1 arrest, almost complete growth inhibition, and morphological changes suggestive of differentiation. Together, these results suggest that silibinin caused hypophosphorylation of Rb-related proteins may in part be responsible for its cancer preventive and anti-carcinogenic efficacy in different cancer models including PCA.

Key Words: silibinin, retinoblastoma, cell cycle, CDKI, CDK, cyclin

1. Introduction

Prostate cancer (PCA) is the second most common invasive malignancy and leading cause of cancer-related deaths in American men [1]. Recent studies have shown that naturally occurring dietary agents and those consumed as dietary supplements could be effective against several human malignancies including PCA [2-4]. In this regard, flavonoid class of phytochemicals has been shown to be both preventive and therapeutic agents against various malignancies [5]. One such naturally occurring flavonoid is silibinin isolated from milk thistle. Silibinin and its crude form silymarin are used clinically as anti-hepatotoxic agents [6], and are consumed as dietary supplement around the world. Both silibinin and silymarin are well tolerated and largely free of adverse effects [7]. Several recent studies by us and others have shown the cancer preventive and therapeutic efficacy of silibinin and silymarin in different animal tumor models and cell culture systems [7-10].

Eukaryotic cell growth is governed by cell cycle progression where a growth signal "turns-on" an interaction between cyclin-dependent kinases (CDKs) and cyclins leading to phosphorylation of retinoblastoma (Rb) and related proteins (e.g. Rb/p107, Rb2/p130) [11,12]. This event releases transcription factor E2Fs, from their Rb-complexes, for growth and proliferation [11,12]. Conversely, a growth inhibitory signal induces the expression of CDK inhibitors (CDKIs) that prevents CDK-mediated Rb phosphorylation keeping E2F bound to Rb and causing growth inhibition [11,12]. The Rb family of proteins, Rb/107, Rb/p110 and Rb2/p130, cooperate to regulate cell cycle progression through G1 phase of the cell cycle [11,12]. Hyperphosphorylated Rb (ppRb) exerts most all of its effects in a defined frame of time during first 2/3rd of the G1 phase of the cell cycle [13]. During this time frame, mammalian cells make most of their decision about growth versus quiescence [13]. However, the

phosphorylation status of each of the Rb family members differs throughout the cell cycle [14]. It has been hypothesized that Rb must be phosphorylated and inactivated by a CDK-cyclin complex in the cells to allow for cell cycle progression. In cycling cells, an activated complex of G1 phase cyclins and CDKs phosphorylates Rb and/or Rb-related proteins, resulting in the release of E2F family of transcription factors for cell growth and proliferation [15-17]. In contrast, CDKIs interfere with cyclin/CDK activity, leading to an increase in hypophosphorylated Rb (pRb) bound to E2F and causing a cell cycle arrest at the G1 checkpoint [18-20].

Since hyperphosphorylation of Rb is a requirement for cell cycle progression and that a blockade of Rb phosphorylation universally inhibits cell cycle progression [16,21,22], here we assessed whether silibinin causes hypophosphorylation of Rb-related proteins as its growth inhibitory response in human PCA cells and whether this effect is via modulation of cell cycle regulators?

2. Materials and methods

2.1. Cell line and reagents

Human prostate carcinoma DU145 cells were from American Type Culture Collection (Manassas, VA). RPMI 1640 medium and all other culture materials were from Life Technologies, Inc. (Gaithersburg, MD). Silibinin was from Sigma Chemical Co. (St. Louis, MO), and its purity was checked as 100% [10]. Anti-Cip1/p21 antibody was from Calbiochem (San Diego, CA), and anti-Kip1/p27 was from Neomarkers (Fremont, CA). The primary antibodies for cyclin D1, cyclin E, CDK2, CDK4, Rb/p107, Rb2/p130, E2F3, E2F4, E2F5, rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibody used in this study were purchased from Santa Cruz

Biotecnology, Inc. (Santa Cruz, CA). Anti-p19 antibody was from PharMingen (San Diego, CA), and anti-p15, p16 and p18 antibodies were from Upstate Biotechnology (Lake Placid, NY). The ECL detection system was from Amersham (Arlington Heights, IL).

2.2. Cell culture, silibinin treatment and Western blot analysis

DU145 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin-streptomycin under standard culture conditions. To assess the effect of silibinin on the levels of different cell cycle regulators, 60% confluent cultures were treated with either dimethyl sulfoxide (DMSO) alone or different doses of silibinin (50, 100 or 200 μM) in DMSO for 6, 12 and 24 hrs. The final concentration of DMSO in culture medium during silibinin treatment did not exceed 0.1% (v/v), and therefore, same concentration of DMSO was present in control dishes. After these treatments, medium was aspirated, cells were washed two times with cold PBS, and cell lysates were prepared as described in detail recently [9,10]. For western immunoblotting, 40-60 μg of protein lysate per sample was denatured with 2x-sample buffer, samples were subjected to SDS-PAGE on 6, 12 or 16% gel, and separated proteins were transferred onto membrane by Western blotting. The levels of Rb/p107, Rb2/p130, E2F3, E2F4, E2F5, CDK2, CDK4, cyclin D1, cyclin E, Cip1/p21, Kip1/p27, p15, p16, p18 and p19 were determined using specific primary antibodies, followed by peroxidase-conjugated appropriate secondary antibody and ECL detection.

Autoradiograms of the immunoblots were scanned using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). The blots were adjusted for brightness and contrast for minimum background, and the mean density for each band was analyzed using Scanimage Program (National Institutes of Health, Bethesda, MD). The densitometric data shown under the

immunoblots are arbitrary units. Unless specified otherwise, the results showed in each case are from one to three independent experiments.

2.3. Cell cycle analysis

DU145 cells at 60% confluency were treated with either DMSO alone or varying concentrations of silibinin. After 24 hrs of treatments, medium was aspirated, cells were quickly washed two times with cold PBS and trypsinized, and cell pellets were collected. Approximately 0.5×10^6 cells in 0.5 ml of saponin/propidium iodide (PI) solution (0.3 % saponin, 25 μg/ml PI, 0.1mM EDTA and 10 μg/ml RNase in PBS) were incubated at 4^0C for 24 hrs in the dark. Cell cycle distribution was then analyzed by flow cytometry using the FACS analysis core service of the University of Colorado Cancer Center (Denver, CO).

2.4. Cell growth assay and cell morphological analysis

DU145 cells were plated at 5000 cells/cm^2 density in 35 mm-dishes under the standard culture conditions. After 24 hrs, cells were fed with fresh medium and treated with either DMSO alone or different concentrations of silibinin. After 2, 4 and 6 days of these treatments, cells were trypsinized, collected, and counted using a haemocytometer. Trypan blue dye exclusion was used to determine cell viability. For morphological changes, DU145 cells were grown in 100-mm dishes, and at 45-50% confluency were treated with DMSO alone or 100 and 200 μM concentrations of silibinin. After 24 and 48 hrs of these treatments, pictures were taken using phase-contrast microscope at x200 magnification.

3. Results and discussion

Rb and related proteins (Rb/p107, Rb2/p130) are one of the main regulators of the G1-S transition [16]. Since Rb itself is mutated and nonfunctional in DU145 cells [23], we studied the effect of silibinin on both hyper- and hypo-phosphorylation of Rb-related proteins. Western blot analysis showed that silibinin treatment of DU145 cells resulted in a strong increase in hypophosphorylated forms of Rb/p107 and Rb2/p130 (Fig. 1). In case of Rb/p107, as compared to DMSO treated control, 6 hrs silibinin treatment caused 1.7-2.3-fold increase in the levels of hypophosphorylated Rb/p107 (Fig. 1A). Other time periods of silibinin exposure also showed moderate increase, however, maximum effect was evident at 6 hrs (Fig. 1A). In case of Rb2/p130, silibinin treatment resulted in 1.4-2.3-fold increase in its hypophosphorylated form after 6 hrs of treatment, which persisted during longer treatment time of 12 and 24 hrs (Fig. 1B). Silibinin treatment, however, did not show any noticeable change in protein expression of E2F3, E2F4 and E2F5. Together, these results suggest that the observed induction in hypophosphorylated levels of Rb-related proteins by silibinin could possibly be due to an upstream growth inhibitory signal that changes phosphorylation status of Rb-related proteins but is unresponsive to the expression of E2Fs.

An induced level of CDKIs has been shown to result in an increase in its binding with CDK-cyclin complex that ultimately decreases the kinase activity of CDKs towards the phosphorylation of Rb and related proteins keeping them in hypophosphorylated forms [24-27]. Accordingly, we next assessed whether induction of hypophosphorylation of Rb/p107 and Rb2/p130 by silibinin was associated with the alteration in cell cycle regulatory molecules. Treatment of DU145 cells with different concentrations of silibinin resulted in strong induction of Cip1/p21, Kip1/p27 and p15 protein levels (Fig. 2). The densitometric analysis of the blots for Cip1/p21 showed that 6, 12 and 24 hrs silibinin treatments resulted in approximately 3-, 8- and

13-fold induction, respectively (Fig. 2A). Similar to its effect on Cip1/p21 induction, treatment of cells with silibinin also showed an up-regulation of Kip1/p27 protein levels. However, in this case, maximum induction (~6-fold) was evident after 12 hrs of treatment (Fig. 2B). In the studies assessing the effect of silibinin on INK family of CDKIs, p18 levels did not change following silibinin treatments at different doses and time periods. In case of p15, silibinin treatment showed up to ~2-fold induction in its protein levels following 24 hrs of treatment (Fig. 2C).

In the studies assessing the effect of silibinin on the protein levels of CDKs and cyclins, it showed strong decrease in the expression of CDK4 and CDK2 levels (Fig. 3). The densitometric analysis of the blots for CDK4 protein levels showed that compared to vehicle control, silibinin treatment for 24 hrs resulted in up to 90% decrease (Fig. 3A). In case of CDK2, however, maximum effect of silibinin was evident after 12hrs treatment accounting for up to 70% decrease (Fig. 3B). Unlike its effect on CDKIs and CDKs expression, silibinin treatment of DU145 cells did not result in any change in cyclin D1 and cyclin E levels.

Based on above findings, next we examined the effect of silibinin on cell cycle progression. FACS analysis of DMSO treated control and silibinin treated DU145 cells clearly showed that silibinin induces a strong G1 arrest in cell cycle progression (Fig. 4A). The observed effect of silibinin was dose-dependent where compared to DMSO control showing 52% cells in G1, silibinin treatment of cells at 50, 100 and 200 μM doses for 24 hrs resulted in 54, 65 and 68% cells in G1 phase, respectively (Fig. 4A). The effect of silibinin on G1 arrest was largely accompanied by a decrease in S phase cells whereas G2/M population remained unchanged (Fig. 4A). In the studies assessing the effect of silibinin on cell growth, it resulted in a highly significant to complete growth inhibition ($P < 0.001$) in both dose- and time-dependent manner. Compared to DMSO controls, 2 days of silibinin treatment at 50, 100, 150 and 200 μM doses

resulted in 41, 43, 54 and 63% inhibition, respectively (Fig. 4B). A much stronger inhibitory effect of silibinin on DU145 cell growth was observed after 4 and 6 days of treatments where 100, 150 and 200μM doses showed complete growth inhibition (Fig. 4B).

In the studies analyzing the biological fate of silibinin treated cells following their almost complete growth inhibition cmpared to control, silibinin did not result in noticeable cell death (data not shown), but induced morphological changes suggestive of differentiation (Fig. 4C). Whereas lower doses of silibinin and shorter treatment time were also effective in showing morphological changes, 200 μM dose of silibinin and 48 hrs treatment time induced strong morphological changes. In this case, compared to vehicle treated control cells that were small in size with normal cell morphology, silibinin treated cells became larger in size and their shape was elongated with prominent dendrite like structures that were connected with other nearest cells (Fig. 4C).

An identification of regulatory mechanism controlling cell cycle progression and cell growth has been extremely useful in developing targeted approaches for cancer prevention and therapy [28,29]. Cell division in eukaryotic cells is controlled by sequential activation of a family of serine-threonine protein kinases known as CDKs that regulate cell cycle progression through various key transitions. Two distinct CDKs, CDK4 (or CDK6) and CDK2, control G1 progression and entry into S phase. Inactivation of either kinases leads to cell cycle arrest and withdrawal from the mitotic cycle [30]. In recent years, a growing number of proteins, collectively named as CDKIs, have been characterized as negative regulators of CDKs in G1 phase [18-20]. A previous study from our group showed that silymarin induces the expression of CDKIs and inactivation of kinase activity of CDKs and associated cyclins [10]. In this study, using pure compound silibinin we identified down-stream effector in cell cycle regulatory machinery, the phosphorylation status of Rb-related proteins in DU145 cells. The data obtained

convincingly suggest that silibinin induces hypophosphorylation of Rb/p107 and Rb2/p130 proteins that is via an upstream induction of CDKI levels and a decrease in CDK expression. Together, these results suggest that initially silibinin induces a growth inhibitory signal that causes up-regulation of CDKIs as well as a decrease in CDKs. Collectively, these events impair the kinase activity of CDKs towards the phosphorylation of Rb-related proteins leading to an increase in their hypophosphorylated forms and thereby growth inhibitory response.

In a controlled cell cycle progression, DNA synthesis begins with CDK4 and/or CDK6 mediated phosphorylation of Rb and/or related proteins that are complexed with E2F family of transcription factors. Several studies have shown the critical role of Rb family of proteins in controlling G1 checkpoint of the cell cycle and regulating G1-S phase transition [16]. Rb and its related proteins, p107 and p130, bind to various members of the E2F family preventing the transcriptional activity of E2Fs. However, hyperphosphorylation of Rb, p107 and/or p130 by CDKs dissociates these proteins from E2F, and free E2F mediates cell cycle progression by activating the genes required for S phase [31]. In cancer cells, Rb is often mutated that leads to the loss of its tumor suppression function, and as a result, both proliferation and apoptotic machinery are deregulated [32]. Consistent with several other cancer cells, human prostate carcinoma DU145 cells are also Rb deficient [23], and therefore, the cell cycle progression in these cells is possibly regulated by Rb/p107 and Rb2/p130 proteins. Accordingly, our results showing that silibinin induces hypophosphorylation of these two Rb-related proteins could be critical in defining the G1 arrest, and cell growth inhibitory and differentiation inducing effects of silibinin in human prostate carcinoma DU145 cells.

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FIGURES

FIGURE LEGENDS

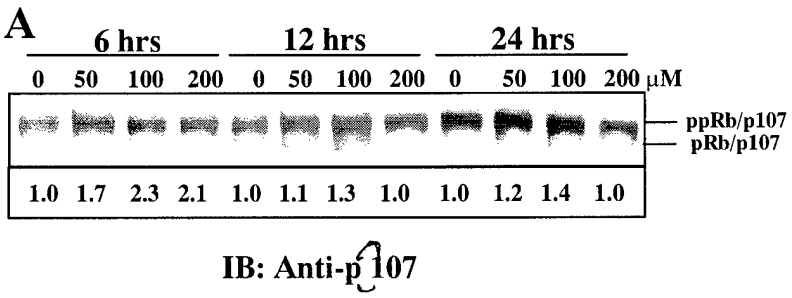
FIG. 1. Silibinin induces hypophosphorylation of Rb/p107 and Rb2/p130 in human PCA DU145 cells. Cells were cultured as described in Methods, and treated with either vehicle alone or varying concentrations of silibinin for 6, 12 and 24 hrs as described in Methods. At the end of these treatments, total cell lysates were prepared, and subjected to SDS-PAGE on 6% gels followed by Western blotting as described in Methods. Membrane was probed with anti- **(A)** Rb/p107 or **(B)** Rb2/p130 antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone. ppRb/p107, hyperphosphorylated Rb/p107; pRb/p107, hypophosphorylated Rb/p107; ppRb2/p130, hyperphosphorylated Rb2/p130; pRb2/p130, hypophosphorylated Rb2/p130. IB, immunoblotting.

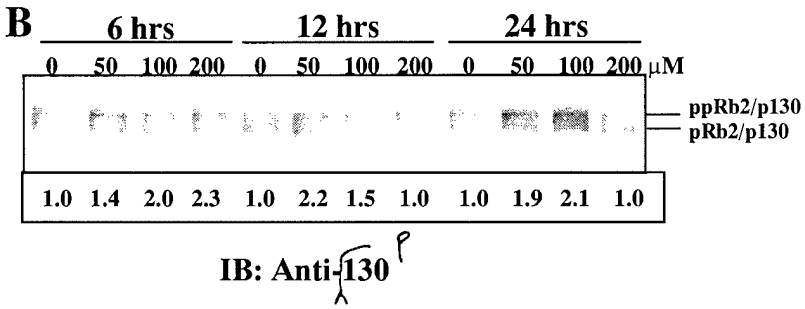
FIG. 2. Silibinin induces CDKI protein levels in human PCA DU145 cells. Cells were cultured as described in Methods, and treated with either vehicle alone or varying concentrations of silibinin for 6, 12 and 24 hrs as described in Methods. At the end of these treatments, total cell lysates were prepared, and subjected to SDS-PAGE on 16% gels followed by Western blotting as described in Methods. Membrane was probed with anti- **(A)** Cip1/p21, **(B)** Kip1/p27, or **(C)** p15 antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone.IB, immunoblotting.

FIG. 3. Silibinin decreases CDK protein levels in human PCA DU145 cells. Cells were cultured as described in Methods, and treated with either vehicle alone or varying concentrations of silibinin for 6, 12 and 24 hrs as described in Methods. At the end of these treatments, total cell lysates were prepared, and subjected to SDS-PAGE on 12% gels followed by Western blotting as

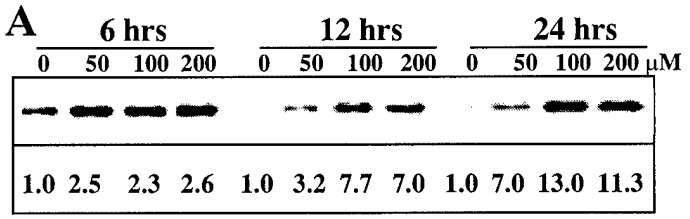
described in Methods. Membrane was probed with anti- **(A)** CDK4 or **(B)** CDK2 antibody followed by peroxidase conjugated appropriate secondary antibody and visualization by ECL detection system. Different treatments are as labeled in the figure; lanes labeled as **0** denote DMSO treatment alone. IB, immunoblotting.

FIG. 4. Silibinin exhibits G1 arrest, growth inhibition and differentiation of human PCA DU145 cells. **(A)** Cells were cultured as described in Methods, and treated with either vehicle alone (control) or varying concentrations of silibinin for 24 hrs. At the end of these treatments, cells were collected, incubated with saponin/PI solution and subjected to FACS analysis as detailed in Methods. **(B)** Cells were plated in 35 mm dishes, treated with vehicle (control) or different concentrations of silibinin, and after 2, 4 or 6 days cells were counted as detailed in Methods **(C)**. Cells were grown in 100 mm dishes, and treated with DMSO (control) or 200 μM silibinin and after 48 hrs, pictures were taken using phase-contrast microscope at x200 magnification.





Tyagi et al, Figure 1



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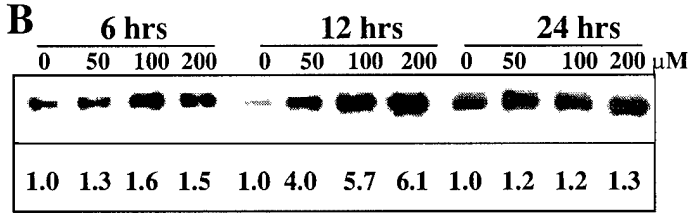

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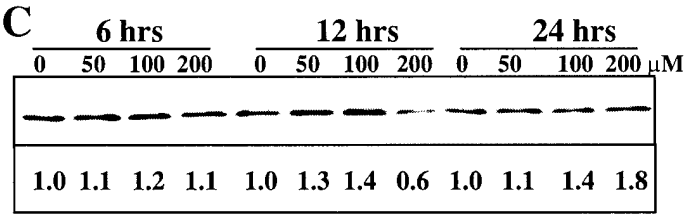
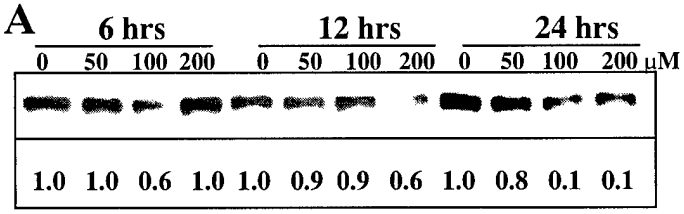
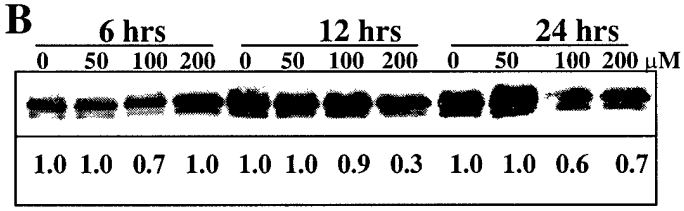


IB: Anti-p15

Tyagi et al, Figure 2

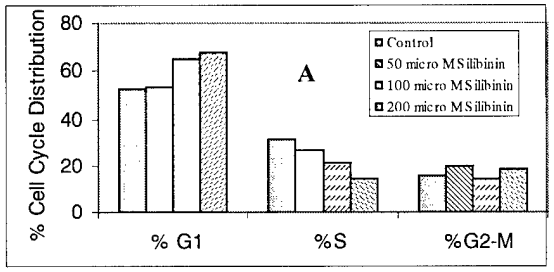


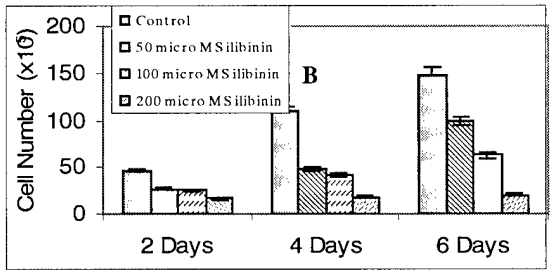
IB: Anti- CDK4

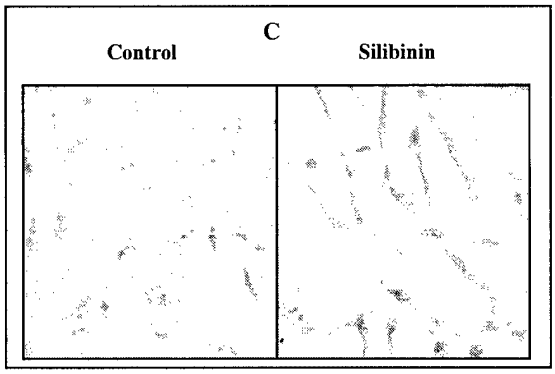


IB: Anti- CDK2

Tyagi et al, Figure 3







Tyagi et al, Figure 4